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THE RELATION BETWEEN TOXIN PRODUCTION AND PROTEIN SYNTHESIS BY *CORYNEBACTERIUM DIPHTHERIAE*

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Until now the preparation of diphtheria toxin has been influenced chiefly by the wish to obtain maximal toxin yields. The technical progress of the preparation of the various mediums is a striking example of this. About 20 years ago a final product containing 10 to 20 Lf per ml was considered a satisfactory one. Since then these values have risen to 80 to 100 Lf per ml. The production of diphtheria toxin is closely connected with the metabolism of sugars and nitrogenous material present in the medium. Some of the amino acids originally present in the medium disappear during the growth period, whereas others not present at first are synthesized by *Corynebacterium diphtheriae*. Di- and polypeptides are also produced (see Linggood and Woiwod,[1] Proom and Woiwod[2] and Woiwod[3]). In addition, Norlin[4] has shown that nonspecific proteins, as well as specific toxin proteins, appear. Lawrence and Pappenheimer[5] have demonstrated in three detailed studies that the undesirable reactions of older children and adults to toxoid injections are mainly due to nonspecific proteins present in the vaccine, which together with the specific toxin protein are produced by the diphtheria bacilli and are therefore also found in the toxoid. They designated the undesirable nonspecific proteins, which should be removed in order to purify the toxoid, as "P factor."

The principal point in the various purification processes is separating the protein material. The nonprotein nitrogen (from peptones, di- and polypeptides, etc.) can be very easily removed by ultrafiltration or salting out with ammonium sulphate. Therefore the only point of interest lies in the presence of protein nitrogen. However, the specific and nonspecific proteins formed by *C. diphtheriae* during growth have few physicochemical differences, and their separation can therefore be brought about only with great difficulty. In the present study no attempt was made to separate the specific toxin protein and the nonspecific protein.

With respect to the incubation time, there is no agreement among the various authors. Most workers are guided by maximal toxin production and not the quality of the toxin formed as an objective. Amies[6] calls attention to the fact that together with toxin, several other, less desirable metabolites are formed during growth and that attention should therefore be given to the incubation time. Holt[7] gives an incubation time of 6 days for his semisynthetic medium. Pope and Smith[8] incubate their cultures

Received for publication November 28, 1949.

1. Linggood, F. V. and Woiwod, A. J. 1948, Brit. J. Exper. Path. **29**: 283.
2. Proom, H. and Woiwod, A. J. 1949, J. Gen. Microbiol. **3**: 319.
3. Woiwod, A. J. 1949, J. Gen. Microbiol. **3**: 312.
4. Norlin, G. 1947, Acta path. et microbiol. Scandinav. **24**: 525.
5. Lawrence, H. S. and Pappenheimer, A. M., Jr. 1948, Am. J. Hyg. **47**: 226, 233, 241.
6. Amies, C. R. 1948, J. Path. & Bact. **60**: 595.
7. Holt, L. B. 1948, Brit. J. Exper. Path. **29**: 335.
8. Pope, C. G. and Smith, M. L. 1932, J. Path. & Bact. **35**: 573.

for 10 days. Pappenheimer[9] incubates his casein hydrolysate medium for 7 days. Pappenheimer and Johnson[10] use an incubation time of 6 days for a gelatine hydrolysate medium. Linggood and Fenton[11] grow their "normal" cultures for 10 days but harvest their "shaking" cultures after 48 to 96 hours. Linggood and Woiwod[1] recommend an incubation of 10 days.

TABLE 1.—*Determinations made on each batch of diphtheria culture. Batch no. 657.*

Days incubated	Lf/ml	Percent total nitrogen	Percent protein nitrogen	Lf/mg total nitrogen	Lf/mg protein nitrogen
0		0.318			
1					
2	12	0.303	0.011	4.0	109
3	31	0.294	0.010	10.5	310
4	52	0.290	0.010	17.9	520
5	70	0.283	0.012	24.4	580
6	81	0.285	0.013	28.2	600
7	81	0.281	0.014	28.6	580
8	82	0.290	0.021	28.3	390
9	82	0.290	0.022	28.3	360
10	86	0.285	0.025	30.0	350
11	85	0.291	0.024	29.2	350
12	80	0.292	0.022	27.6	360
13	88	0.290	0.026	30.0	340
14	80	0.290	0.027	27.8	300

In the present study we attempted to determine the most favorable time for harvesting in order to obtain the best results, i.e., the moment of the growth period at which toxin content is at a maximum and the protein content at a minimum in the culture medium. Thus, a culture containing this optimal ratio between toxin and protein content would provide the most suitable material for toxoid production, as the nonspecific protein causing undesirable reactions would be at a minimum.

TABLE 2.—*Optimum incubation times of 9 different batches of diphtheria cultures.*

Batch no.	Lf/mg protein nitrogen on different days					Optimum incubation time in days
	4	5	6	7	8	
657	520	580	600	580	390	6
658	340	630	650	400	460	6
659	570	730	820	910	590	7
660	610	700	580	560	470	5
661			520	440		6
662			320	260		6
663			280	295		7
665		539	419	394		5
666	380	425	343			5

EXPERIMENTAL

Though all strains used for the preparation of diphtheria toxin are subcultures of the original PW-8 strain isolated by Park and Williams in 1896,[12] considerable differences, designated by special names or numbers of the respective laboratories, exist among the different strains. Amies[8] recently demonstrated that *C. diphtheriae* may undergo considerable variation. The strain used was a culture obtained from the Wellcome Physiological Research Laboratories at Beckenham, labelled "CN 2000." The incubation temperature was 34 to 35 C.

TABLE 3.—*Comparison of Lf/ml after optimal incubation period and after 10 days incubation.*

Batch no.	Optimum incubation		Lf/ml after 10 days incubation
	Lf/ml	Incubation time in days	
657	81	6	86
658	85	6	92
659	100	7	100
660	80	5	95

The selection of medium is important as the mutually related formation of specific toxin protein and nonspecific protein depends on the composition of the medium. With regard to this, it must be observed that for the routine production of diphtheria toxin on a large scale, relatively simple and inexpensive mediums must be used. The expensive, purely synthetic mediums are generally of no use for wholesale production. In the National Institute for Public Health at Utrecht, the so-called "Pope medium" is used. It was described for the first time by Pope and Smith[8] and has since then been applied with slight modifications. This medium consists mainly of broth obtained by tryptic digestion of horse muscle, to which are added as growth factors the vitamin B complex derived from yeast, further lactate, citrate, acetate, iron and maltose.

Every day the contents of a number

9. Pappenheimer, A. M., Jr. 1947, J. Biol. Chem. **167:** 251.
10. Pappenheimer, A. M. Jr. and Johnson, S. J. 1947, Brit. J. Exper. Path. **28:** 239.
11. Linggood, F. V. and Fenton, E. L. 1947, Brit. J. Exper. Path. **28:** 354.
12. Park, W. H. and Williams, A. W. 1896, J. Exper. Med. **1:** 164.

of culture flasks (usually five) were mixed, and after filtration the following values (in percentages) of this mixture were determined: (1) toxin content in flocculation units per ml (Lf/ml), (2) total nitrogen and (3) protein nitrogen. From this it was possible to calculate Lf/mg total nitrogen and Lf/mg protein nitrogen. A typical example is given in table 1 and partly shown in figure 1.

From the figure and the table the following may be concluded. The total nitrogen content of the broth decreases during growth. This is due to the formation of cell proteins by the bacteria filtered off before analysis. The protein nitrogen slowly increases during the entire period of growth, while the Lf value reaches a maximum during growth. From this it follows that the value of Lf/mg protein nitrogen will at a certain moment after the inoculation reach a definite maximum and decrease thereafter, but this maximum need not coincide with maximal toxin production. In our first experiment the optimal relation between Lf and protein nitrogen was reached after 6 days incubation.

We have determined the optimal incubation time for a number of diphtheria cultures by repeating this experiment, in each case with essentially the same results (table 2). Our results show that the optimal value of the Lf/mg protein nitrogen ratio is reached long before the conventional incubation time of 10 days, and that this optimum usually does not coincide with maximal toxin production (table 3).

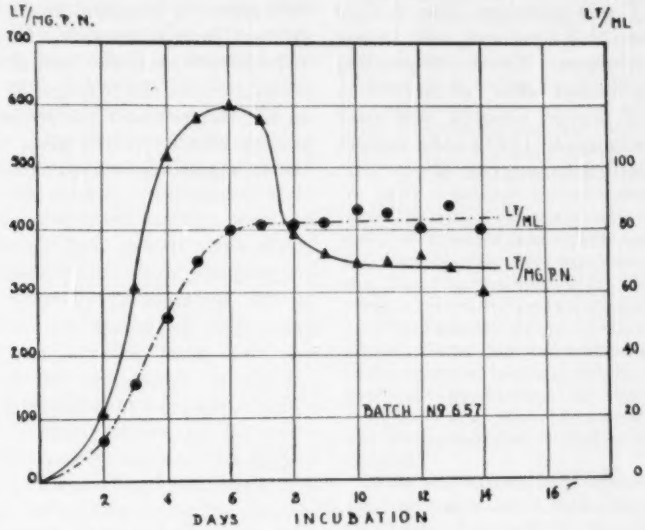

FIG. 1.—Graph of Lf/mg protein nitrogen and Lf/ml in batch no. 657 (cultures of *C. diphtheriae*).

In order to obtain an optimal degree of purification of the toxoid, the toxoid should be made from toxin in which the ratio Lf/mg protein nitrogen is maximal. In other words, the toxin used should contain as few nonspecific proteins as posssible. To obtain this, a nonmaximal toxin content must often be accepted.

To demonstrate that the ratio Lf/mg protein nitrogen is conclusive for the

purification of the toxoid to be obtained from the toxin, the following example is given:

Toxin P 638 contained after 10 days incubation 66 Lf/ml and 350 Lf/mg protein nitrogen. After ultrafiltration of the toxoid derived from this toxin, the last value was 550 Lf/mg protein nitrogen, and after the final salting out 860 Lf/mg protein nitrogen with a recovery of 75%.

Toxin P 660 contained after 6 days incubation 72 Lf/ml and 650 Lf/mg protein nitrogen. The corresponding toxoid contained after ultrafiltration 720 Lf/mg protein nitrogen, and after the final salting out 1320 Lf/mg protein nitrogen with a recovery of 72%.

SUMMARY AND CONCLUSIONS

It has been shown that most undesirable reactions to diphtheria toxoid are due to the nonspecific proteins formed in the culture medium during growth of *Corynebacterium diphtheriae*. Since the specific toxin protein and the nonspecific protein are difficult to separate, an attempt was made to obtain cultures containing a maximum amount of toxin with a minimum amount of protein formation. A maximal ratio of toxin content/protein content (Lf/mg protein nitrogen) was obtained after 6 days incubation on the average, in contrast to the conventional 10-day incubation period, which yields a lower value for the Lf/mg protein nitrogen ratio.

STUDIES ON THE EXPERIMENTAL EPIDEMIOLOGY OF RESPIRATORY INFECTIONS

I. AN APPARATUS FOR THE QUANTITATIVE STUDY OF AIR-BORNE RESPIRATORY PATHOGENS

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The fundamentals basic to the development of an apparatus capable of adequately studying air-borne infections include: (1) the production of an aerosol, (2) its assay both as to microbial content and infectivity and (3) the necessary safety devices incidental to operation. This paper describes such an apparatus, developed during the period 1942 to the present, so designed as to allow for increased safety, ease of manipulation and quantitative study of extremely pathogenic microorganisms. Various instruments for the experimental production of respiratory infections in animals have been described in the past which satisfy the basic requirements to a greater or lesser degree. A short review of the more significant developments in this field of instrumentation therefore seems appropriate before describing the apparatus in use at our laboratories.

Martini[1] described an apparatus in which the infectious material was atomized into a chamber within which the experimental animals were held in a screen cage. Air from a hand-operated pump was supplied to a "Parolin" atomizer which seemed to be a right angle jet atomizer. Effluent air from the chamber was passed through a platinum tube ending in the flame of a gas burner. This apparatus has been used to produce primary pneumonic plague in rats.

Goss[2] built a machine for the purpose of blowing dust, containing plague bacilli, into the air which is breathed by the experimental animals. The head of the animal being exposed was sealed with cotton into a funnel connected to the dust chamber. Infectious material in dust was placed on the floor of the chamber from which it was picked up in a stream of air from a rubber bulb and carried to the experimental animals. Guinea pigs and rabbits were exposed in this apparatus to dust containing both plague bacilli and pneumococci. The organisms were dried in blood or peritoneal exudate and ground with sterile room dust to produce the infectious material.

Lange and Keschischian[3] atomized an infectious suspension into a glass chamber using a direct spray right angle jet type atomizer constructed of glass. Air to the atomizer was supplied from a hand-operated pump. The spray produced numerous droplets of 2 to 20 μ in size and some as large as 2000 μ. The atomizer was directed against a glass wall in order to retain some of the larger particles. The animals were held behind the atomizer in such a manner that only their heads were exposed to the cloud. Excess air was released from the chamber through a cotton filter. No direct determination of the cloud concentration was made, but the lungs of the exposed animals were assayed for their bacterial content. This apparatus has been used

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1. Martini, E. 1901, Ztschr. f. Hyg. u. Infektionskr. **38**: 332–342.
2. Goss, W. 1905, Arch. des Sci. Biol. **11**: 330–350.
3. Lange, B. and Keschischian, K. H. 1924, Ztschr. f. Hyg. u. Infektionskr. **103**: 569–583.

to infect mice with salmonella and streptococci.

Wells and Henle[4] infected mice with influenza virus by exposing them to a cloud of virus established in a 50 cubic foot chamber. The cloud was produced by atomizing a 10% suspension of mouse lung.

Wells and Lurie[5] exposed animals under a glass bell jar to aerosols produced by a refluxing type of atomizer. The concentration of the material in the cloud was determined by collecting a sample in the Wells air centrifuge. Effluent air from the infection chamber and the centrifuge sampler was incinerated before release to the atmosphere. In a later paper, Wells[6] described a modification of this apparatus.

Buchbinder, Solowey and Solotorovsky[7] were able to infect mice with streptococci (Lancefield Group B) which had been recovered from an aerosol established in a chamber designed by Phelps and Buchbinder.[8]

Loosli, Robertson and Puck[9] described the production of influenza in mice by inhalation of atmospheres containing the virus dispersed as fine droplets. A further development of this major study by Robertson, Puck and Wise[10] led to the construction and operation of two identical rooms in which aerosols of bacteria or viruses could be established under controlled conditions of temperature and humidity. Provision was made for the installation of decontaminating apparatus for the effluent air from the rooms. These experimental rooms have been used to determine the effect of various physical and chemical environments upon bacteria and viruses.

Edward, Elford and Laidlaw[11] developed an all glass apparatus for the study of air-borne influenza virus. They sampled the cloud by collecting a portion in a centrifuge tube, adding a small quantity of broth and centrifuging the particles into the broth. The effluent air from the exposure chamber was led through a disinfectant trap before release to the atmosphere. Decontamination following an exposure was accomplished by flushing out the apparatus with air. The aerosol was produced by atomizing the virus suspension in an Aerograph pencil spray. The humidity of the cloud was controlled by causing it to pass over a liquid mixture of known water vapor pressure.

Glover[12] constructed an exposure chamber consisting of a rectangular metal box divided into two sections of equal size. The cloud of infectious material (tubercle bacilli) was produced by atomizing a suspension of the bacilli into one of the chambers using a modified form of the atomizer incorporated in the Collision Inhaler, which produced a fine spray as described by Elford and Van den Ende.[13] The spray was operated for two minutes, and the mist, after allowing the larger particles to settle out, was transferred to the other chamber containing the animals. After the completion of an exposure, the chamber was disinfected by formalin vapor. The exposed mice were stored in closed cubicles to prevent air-borne particles from entering the room. Samples of the cloud were taken on a modified form of the Bourdillon sampler in which the cloud was forced through a slit onto a slide coated with a mixture which retained the organisms.

Young, Zelle and Lincoln[14] described an infection chamber built from a rectangular autoclave in which animals were exposed to an aerosol produced by a stainless steel nebulizer. Concentration of the cloud was determined by withdrawing a measured portion of the cloud through a cotton filter and subsequently eluting the organisms into a known quantity of water. Decontamination after a run was accomplished by flushing with clean air for a five-minute period. The ultimate disposal of the contaminated air was not described. This experimental chamber has been used to determine the respiratory pathogenicity of anthrax spores.

Rosebury et al[15] have given a detailed description of the construction and operation of a cloud chamber and its associated equipment which constitutes a completely enclosed system for

4. Wells, W. F. and Henle, W. 1941, Proc. Soc. Exper. Biol. & Med. **48:** 298.
5. Wells, W. F. and Lurie, M. B. 1941, Am. J. Hyg. **34** (Sec. B): 21–35.
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10. Robertson, O. H., Puck, T. T. and Wise, H. 1946, J. Exper. Med. **84:** 559–567.
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12. Glover, R. E. 1944 Brit. J. Exper. Path. **25:** 141–149.
13. Elford, W. J. and Van den Ende, J. 1942, J. Hyg. **42:** 240–265.
14. Young, G. A. Jr., Zelle, M. R. and Lincoln, R. E. 1946, J. Infect. Dis. **79:** 233–246.
15. Rosebury, T. et al, 1947, Experimental Air-Borne Infection, Baltimore, Williams and Wilkins Co.

exposing and housing experimental animals without risk to the operators of the equipment. The aerosols were produced by a "Chicago" atomizer, an all glass, nonrefluxing, peripheral air, direct spray atomizer. The cloud was subsequently diluted and dried in a mixing chamber by the addition of air of the desired humidity. Fluid capillary impingers were used for sampling the clouds during the exposure of the animals, which were held in screen cages within the exposure chamber. Effluent air from the apparatus was incinerated before release to the atmosphere. Following exposure, the animals were transferred to observation storage chambers. This apparatus was used to study the respiratory pathogenicity of numerous infectious agents.

Rosebury, Meikeljohn, Kingsland and Boldt,[16] using the apparatus described above,[15] studied the disinfection of clouds of meningopneumonitis and psittacosis viruses with triethylene glycol vapor.

Wells[6] developed an apparatus for the quantitative study of air-borne infections in which an aerosol was generated by an atomizer inserted tangentially into a two-liter flask. The cloud was passed through a mixing chamber where room air was allowed to enter, diluting the cloud. The infection chamber consists of a hexagonal metal box with six animal holders capable of holding rabbits attached. Only the heads of the animals were allowed to project into the infection chamber. A rubber gasket around the animals' necks made a tight seal. Effluent air from the apparatus was incinerated before release to the atmosphere. Samples of the cloud were withdrawn from the infection chamber and the number of organisms in a given volume determined by an air centrifuge. At the termination of an experiment, the interior of the apparatus was disinfected superficially by ultraviolet light. This apparatus has been used to produce respiratory tuberculosis in rabbits.

The apparatus which we have evolved embodies features of the various developments cited above and certain changes leading to greater reproducibility, easier working conditions and greater flexibility, which we have found necessary in the study of respiratory pathogens.

16. Rosebury, T., Meikeljohn, G., Kingsland, L. C. and Boldt, M. H. 1947, J. Exper. Med. **85**: 65.

GENERAL DESCRIPTION OF APPARATUS

The apparatus in use at this laboratory consists of a number of closed steel chambers which are modifications of the type used by Reyniers[17] for rearing germ-free animals. All are provided with facilities for ventilating and illuminating the interior, manipulating the apparatus within the chambers and transferring material or animals from one part to another without breaking the mechanical barrier. The entire system is so constructed as to permit sterilization by steam under pressure. Two types of chambers are in use at the present time: those which contain the apparatus for exposing the experimental animals to graded doses of air-borne infectious material (work chambers), and those which hold infected animals for observation (storage chambers). A schematic diagram (fig. 1) shows the connections between the various chambers and the associated apparatus for disposal of the contaminated air. Component parts of the apparatus as designated by letter in the following description refer to this diagram.

Work chamber.—The work chamber (fig. 2) is a horizontal, cylindrical chamber approximately 30 inches in diameter and 6 feet long. Access to the interior is provided through a full diameter flanged door at one end of the chamber. Two observation ports of Pyrex glass are situated on the upper surface. Four glove ports, into which are fitted shoulder length neoprene gloves, provide means of manipulating the contents of the chamber (fig. 3). Ventilation for animals within this unit is provided by withdrawing air from the chamber through a connection to the contaminated air disposal line, which is at a slight negative pressure. A valve

17. Reyniers, J. A. 1943, Micrurgical and Germ Free Technique, Springfield, Ill., Chas. C Thomas.

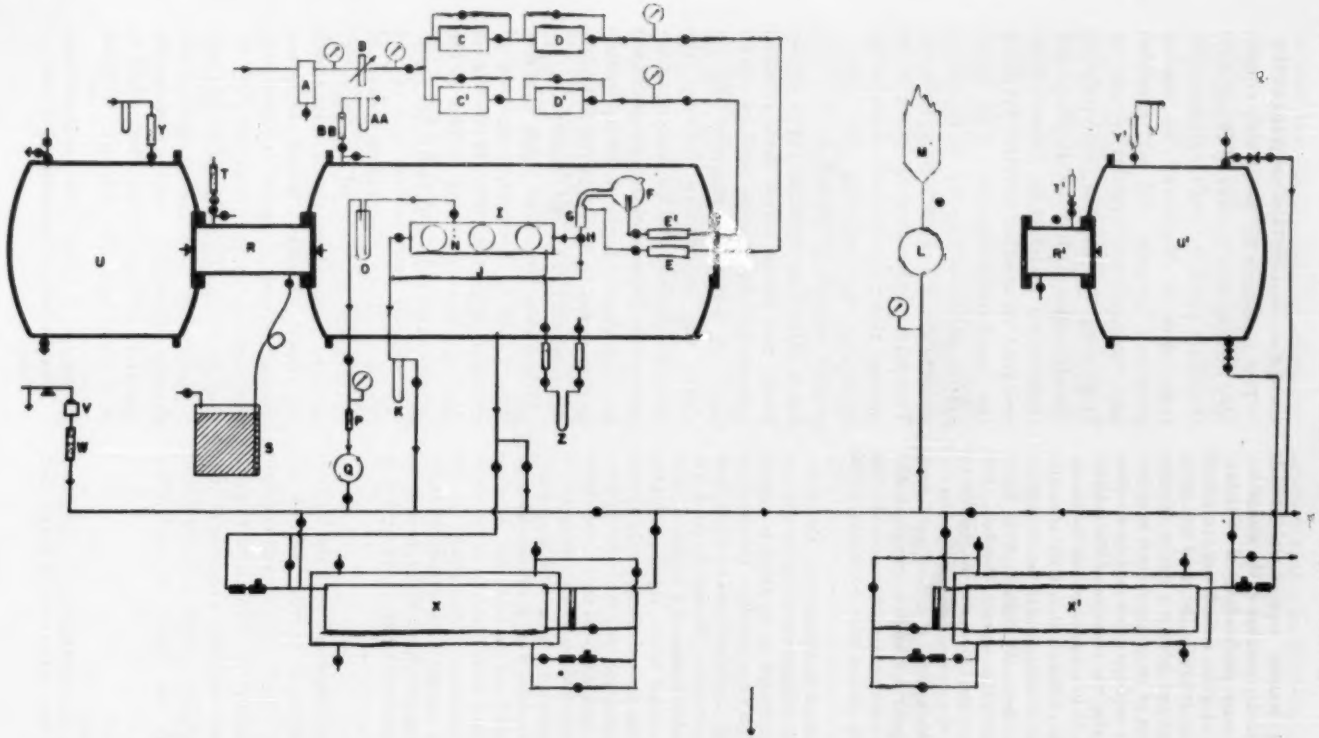

FIG. 1.—Main flow sheet for exposure and storage chambers.

A, Water trap; B, pressure regulating valve; C, air dryer; D, air cooler; E, glass wool filter; F, atomizer; G, mixing chamber; H, three-way valve; I, Infection chamber; J, by-pass line; K, flowmeter; L, water-sealed pump; M, incinerating torch; N, sampling inlet; O, impinger sampler; P, glass wool filter; Q, vacuum pump; R, connecting clave; S, disinfectant storage tank; T, glass wool filter; U, storage unit; V, vacuum regulating valve; W, glass wool filter; X, condensate tank; Y, glass wool filter; Z, water manometer; AA, flowmeter; BB, glass wool filter.

regulates the rate at which air is withdrawn from the chamber. Incoming air from the atmosphere passes through a flow rate meter (AA) and a glass wool filter (BB) before entering the chamber. Numerous piping apertures located at

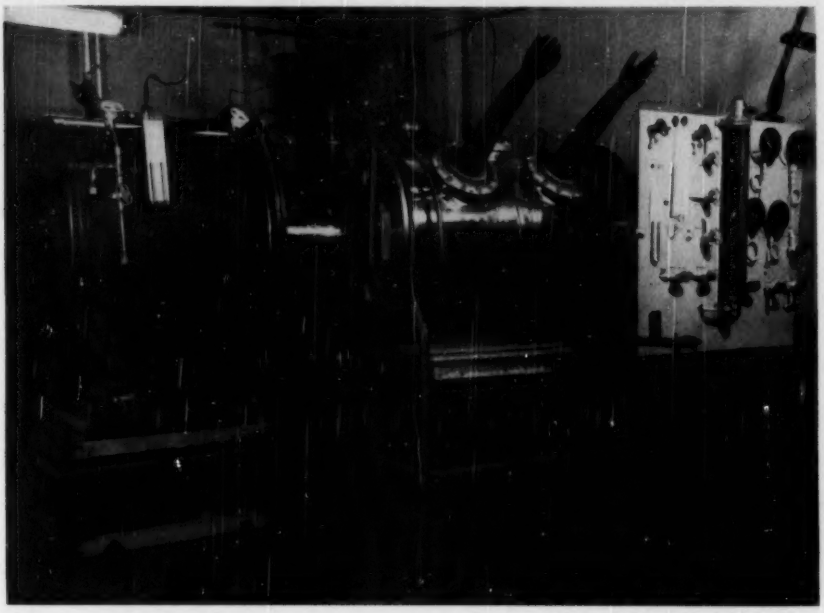

FIG. 2.—Work chamber housing apparatus for exposing animals to air-borne pathogens with attached animal storage unit.

various points on the chamber allow the introduction of steam, air, cooling brine, etc., and removal of waste air from apparatus within the chamber. The temperature within the work chamber is controlled by circulating a glycol-water mixture of the proper temperature through two partial jackets on the outside of the chamber. A refrigerated container within the chamber is used for storage of heat labile materials. The work chamber may be operated as an independent unit or in conjunction with an attached storage unit (fig. 2).

Connecting chambers.—In order to provide a means of coupling various units together, short connecting chambers called "claves" (R) are used (fig. 2). These consist essentially of small cylinders with flange fittings at each end. In use, these claves may be bolted between the larger units. The inside doors (fig. 1, marked by arrows) to the clave are closed and disinfectant run up into the clave through a line entering the bottom of the clave. All of the contaminated air in the clave is displaced upward through a glass wool filter (T). A sight glass mounted in the filter line provides visual indication of complete air displacement. After allowing the disinfectant to act on the contents of the clave for a suitable time, the liquid is drained back into the disinfectant reservoir (S). The clave now may be detached and the large chambers sepa-

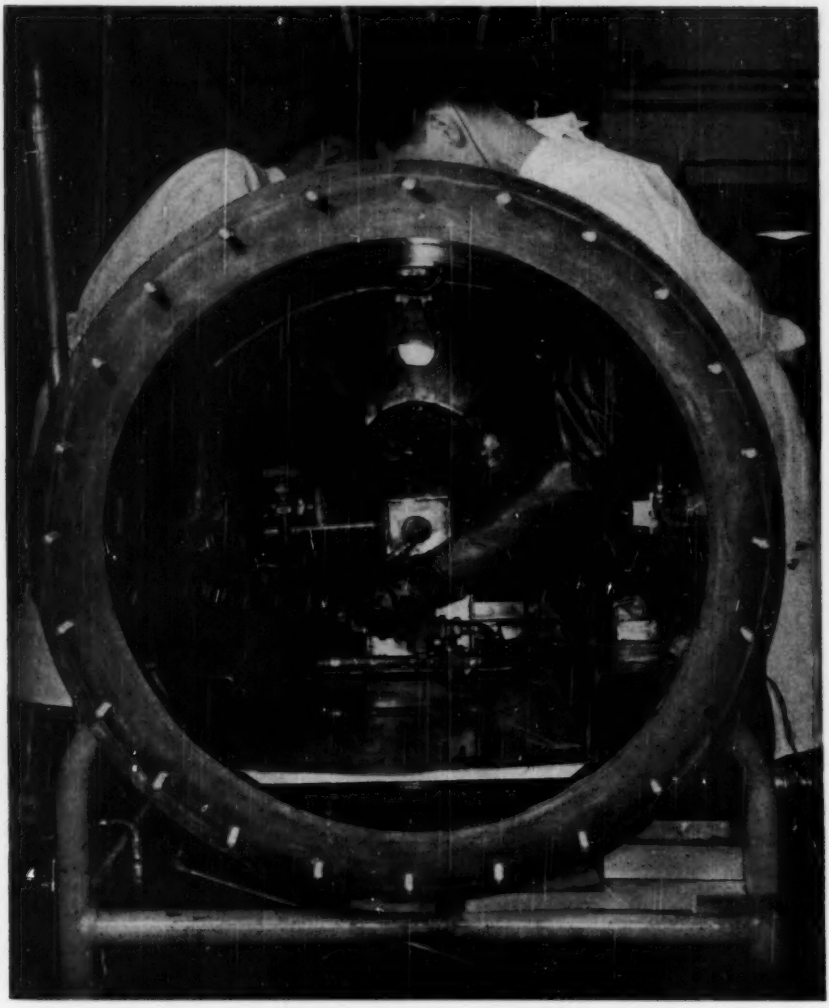

FIG. 3.—Inside of work chamber showing gloves for manipulation of infection system.

rated. Dead animals or other material can be removed from any chamber through an attached clave using a blank flange plate to seal the open end. When material which cannot be decontaminated by immersion in a disinfectant is to be passed through a clave, sterilization can be accomplished by using steam under pressure.

Storage units.—Storage units are used to hold animals for observation following their exposure to infectious clouds (fig. 4). These units are cylindrical chambers 24 inches long and 30 inches

in diameter. A removable, flanged door of the full diameter has an 8-inch opening in its center with a door which can be operated from within the chamber. The storage chambers have one observation port and one glove port on a rolling table to bring them into proper position for attaching to a work chamber or another storage unit. The junctions in the contaminated air disposal line are sterilized with steam prior to breaking the joints.

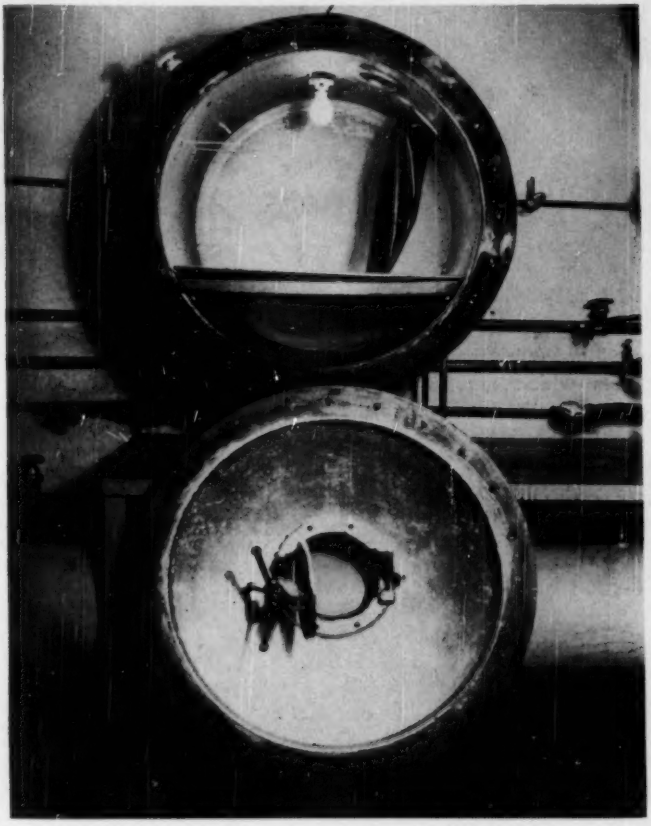

FIG. 4.—Open animal storage unit showing inside door.

through which the contents may be manipulated. Connections for steam, ventilating air, drain and contaminated air disposal lines are provided. A metal screen floor with a catch pan below is used to hold the droppings from the animals. These units can be disconnected from the service lines and moved

Infection system.—The infection system consists of an apparatus for producing an air-borne cloud of infectious material and leading it past the faces of the animals to be exposed. The aerosol is produced by atomizing a suspension of the infectious material in a refluxing, peripheral liquid atomizer (F, fig. 5) of

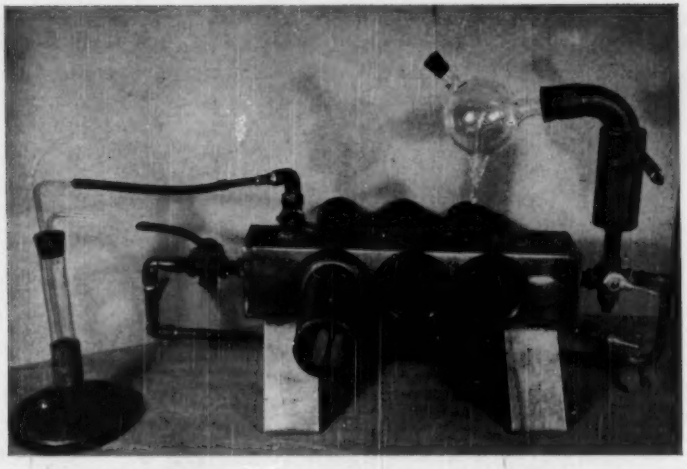

FIG. 5.—Infection system showing atomizer, mixing chamber, animal holders and capillary impinger.

the type used by Wells[18] and DeOme et al.[19] Droplets which are sufficiently minute to become air-borne are carried out of the atomizer with the operating air. In order to provide clean, dry air for operating the infection system, a compressor and storage tank are used. The compressed air is led through an oil trap (A) and a reducing valve (B). The low pressure air is dried by passage through a dryer* (C) and is cooled by passage through a copper coil immersed in a mixture of methyl cellosolve and dry ice (D). The temperature of the air can be controlled by by-passing a portion of the air to the cooler. Before the air is used in the infection system it passes through a glass wool filter (E).

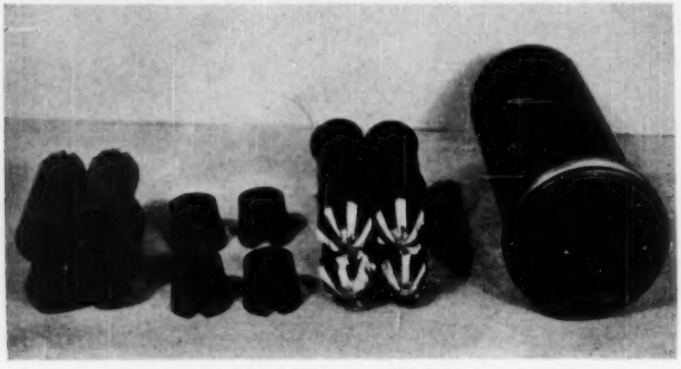

FIG. 6.—Holders for mice, showing four individual holders exposed as a unit.

18. Wells, W. F. 1940, Science, **91**: 172–174.
19. DeOme, K. B. and the Personnel of U. S. Naval Medical Research Unit No. 1, 1944, Am. J. Hyg. **40**: 239–250.

* Pittsburgh Lectrodryer Corp., Pittsburgh, Pa.

The atomizer is operated at a pressure of 20 p.s.i. As the cloud leaves the atomizer it enters a small mixing chamber (G) where secondary air is introduced tangentially into the stream, a procedure found by Henderson[20] to enhance the stability of the cloud. From the mixing chamber, the cloud can be passed either through the infection chamber (I) or through a by-pass line (J) into the contaminated air disposal line. The infection chamber proper (fig. 5) consists of a rectangular metal box with three ports on each side to which animal holders may be attached. Animals are held in lengths of metal tubing of appropriate size (figs. 6 and 7). For guinea pigs, a retaining ring locks around the animal's neck. Mice are held in smaller tubes with a grill at the head end and a stopper at the other. An outer cylinder, closed at one end, fits over each guinea pig holder or four mouse holders and can be hermetically sealed to the ports of the infection chamber. When the holders are in place, only the heads of the animals project into the infection chamber, thereby reducing the amount of coat contamination. The rate of flow of the cloud through the infection chamber is measured by a flow meter (K) in the effluent line after equalizing the pressures within the infection system and the work chamber as indicated by a water manometer

FIG. 7.—Guinea pig holder and exposure tube.

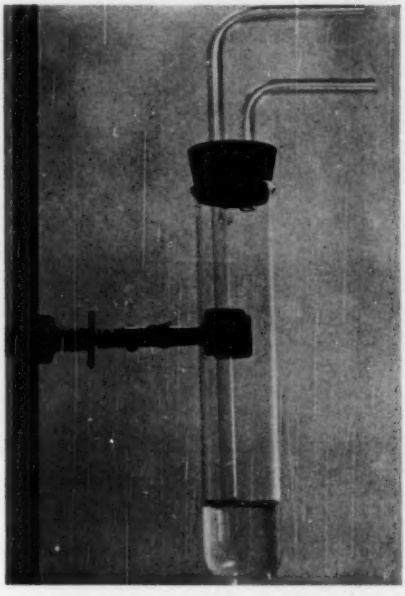

FIG. 8.—Capillary impinger for collection of cloud samples.

20. Henderson, D. W. Personal communication.

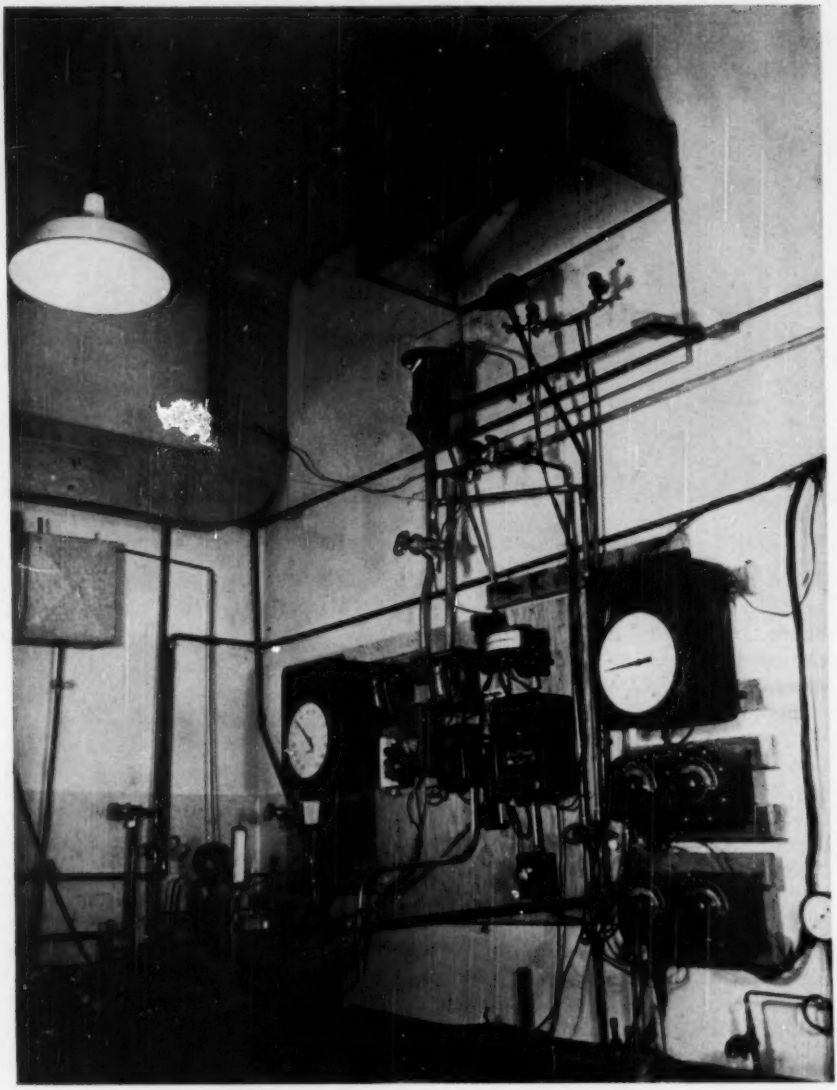

FIG. 9.—Incinerating torch with control equipment for torch and pump.

(Z). The temperature of the infection chamber is indicated by a thermocouple, and the relative humidity of the cloud is determined by measuring the dew-point temperature using an externally cooled mirror located in the effluent line from the infection chamber. A sampling line (N) permits withdrawal of a portion of the cloud to which the animals are being exposed.

Cloud sampling.—The concentration of infectious material in the cloud to which the animals are being exposed is determined by drawing a portion of the cloud through a capillary impinger sampler (O). This sampler (fig. 8)* consists of a large tube containing a measured quantity (20 ml) of liquid through which the portion of the cloud being sampled is led by a length of capillary tubing which dips under the surface of the liquid. The air, after leaving the sampler, passes through a filter (P) to a vacuum pump (Q) and then into the contaminated air disposal system. The vacuum pump applies a negative pressure sufficient to cause the capillary tube to function as a critical orifice. All of the samplers are calibrated before use. Samplers in use at the present time have a flow rate of approximately 5 liters per minute. Knowing the sampling rate, the volume of the sampling fluid and the sampling time, the cloud concentration can be calculated after determining the content of the sampling fluid.

Ventilation and disposal of contaminated air.—To ventilate the chambers, a negative pressure system is used in which air is allowed to pass from the atmosphere, through the unit and into a line maintained at a negative pressure by a pump system. Entering a chamber, the air passes through a filter consisting of a column of glass wool† enclosed in a section of pipe approximately 1 inch in diameter and 10 inches long to prevent the escape of infectious material in the case of an accidental reversal of pressure within the chamber. A disc of wire screen at each end of the filtering column serves to retain the glass wool. The wool in these filters is packed with moderate pressure so as to produce an efficient filter medium without presenting too great a resistance to the flow of the air. The rate of air flow through a unit is measured by a flowmeter in the intake line to the filter. Air is removed from each chamber through a valve into the contaminated air line which is maintained at a negative pressure of 5 inches of mercury by a water sealed pump (L).‡ A pressure regulating valve (V) allows atmospheric air to enter the line as necessary to maintain the desired negative pressure. The contaminated air from the pump is led to an incinerating torch (M) wherein the air is mixed with gas and burned before release to the atmosphere. The incinerating torch (fig. 9) consists of a 12-inch length of 5-inch iron pipe with the bottom end constricted to 1 inch in diameter. The top is fitted with a flange for attaching a cover plate. At a level approximately 6 inches below the top, a perforated steel disc is supported within the torch. On top of this perforated disc there is a 2-inch layer of crushed fire brick. This refractory material is of uniform size, passing a $\frac{1}{4}$-inch sieve but retained by a $\frac{1}{8}$-inch sieve. Below the layer of ceramic material there is a space in which the gas is mixed with the contaminated air from the pump in such proportions that combustion occurs at the upper surface of the refractory material. As the quantity of air passing through the pump is constant, the flame can be adjusted for stable operation.

Pump and torch control system.—In ordinary installations, a water-sealed pump can be operated by running water through the pump, effectively sealing and cooling the rotor. In the installation at this laboratory, however, the sealing water becomes contaminated and must be circulated in a closed system. The

* Copied from a model furnished by Camp Detrick, Frederick, Md.

† Corning #28, basic fiber, Type E, Corning Glass Works, Corning, N. Y.

‡ Nash Hytor Pump, Nash Eng. Co., South Norwalk, Conn.

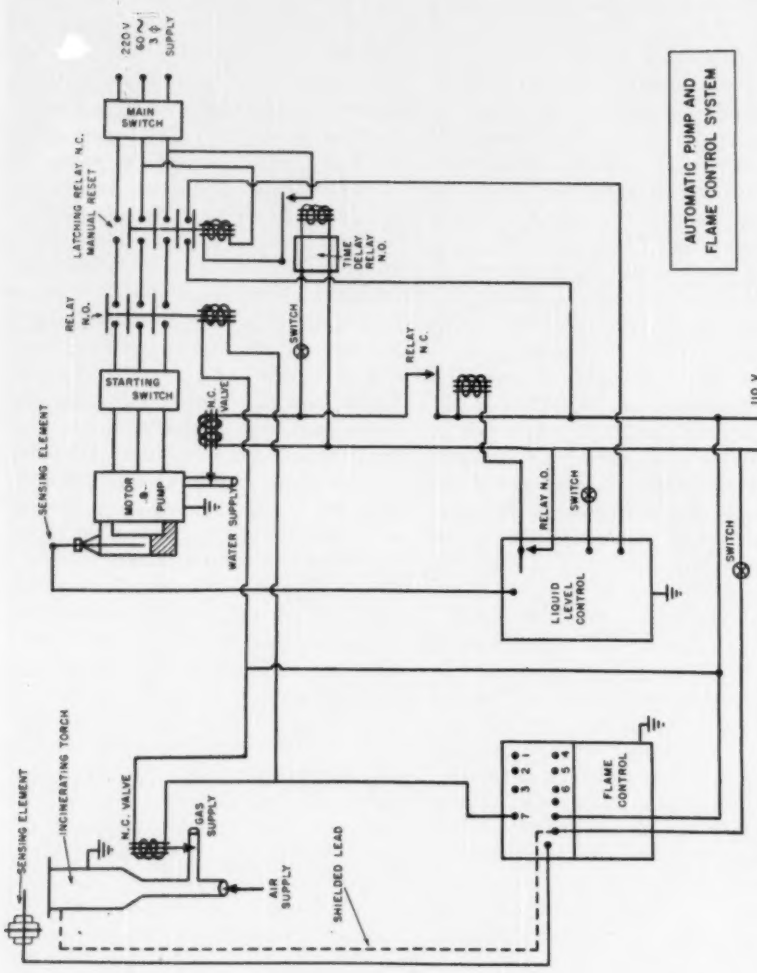

FIG. 10.—Schematic diagram of pump and torch control system.

sealing water is cooled by passage through a liquid-liquid heat exchanger. An electric liquid level control* maintains the proper quantity of water in the pump system. The control circuit (fig. 10) includes relays which stop the pump in the event of an electrical or water supply failure. The incinerating torch is provided with an electronic flame control† arranged so that the pump and the gas supply to the torch are shut off immediately upon failure of the flame.

Sterilization.—All of the chambers described are fitted for sterilization by steam under pressure. Prior to this operation, all glove ports are sealed by flange plates with a valve for control of pressure on the outside of the gloves. As steam is introduced at the top of the chamber, the displaced air passes out through an impulse trap at the bottom of the chamber. The effluent mixture of air and steam from the impulse trap is led through a condensate tank and into the contaminated air disposal system. Steam is directed through all connecting lines of the unit. When all of the air in a unit has been displaced, as indicated by the temperature in the effluent line, the pressure is allowed to increase until the temperature in the effluent line reaches 120 C. Following a suitable period of sterilization, the steam is released and the unit evacuated to approximately 20 inches of mercury to facilitate drying. During the evacuation, the pressure on the outside of the gloves is reduced accordingly. When dry, the unit is opened to the atmosphere and may be cleaned and equipped for the next experiment. The condensate tank into which the chamber drains during sterilization is a cylindrical vessel approximately 5 feet long and 1 foot in diameter with a complete jacket through which either cold water or steam may be circulated. This tank serves to collect the condensate from any unit being sterilized and prevents the entry of steam into the pump system. When the tank is nearly filled, as indicated by a gauge glass, the contents of the tank are sterilized by running steam under pressure into the jacket. Following a suitable period of sterilization, the contents of the tank are drained into the sewer system.

Operation.—The operation of this apparatus can best be described by briefly outlining the steps in a typical experiment. The clean, open work chamber receives all the necessary materials for conducting an experiment, such as sterile atomizers and sampling tubes. Animals that are to be exposed are inserted in their holders and placed in a clean storage chamber which has been stocked with a supply of food and water. After all the necessary material has been placed in the chambers, the suspension of infectious material is placed in the work chamber and the storage chamber attached by means of a connecting clave. After checking the apparatus to see that the proper connections have been made, the chambers are ventilated by opening the valves to the contaminated air line. The suspension of infectious material is now placed in the atomizer, and the flow of atomizing air is initiated with the resulting cloud bypassing the infection chamber. Secondary air is introduced at a rate sufficient to maintain the desired flow through the system. When the correct conditions have been established, the animals are placed in the animal holder covers and sealed to the infection system. The exposure of the animals is started by directing the flow of the cloud through the infection system. During the exposure, samples of the cloud are taken by the

* Trimount Instrument Co., Chicago, Ill.
† Wheelco Instruments Co., Chicago, Ill.

method described, and the temperature and humidity of the cloud are noted. The exposure is stopped at the proper time by directing the cloud through the by-pass line. The animals are removed from the holders and passed through the connecting clave into the storage unit. Additional animals are exposed in a similar manner. At the termination of an experiment articles which must be removed immediately are placed in the connecting clave. The doors to the clave are closed tightly and disinfectant run into the clave. After a period of disinfection, usually 10 minutes if only glass or metal objects are in the clave, the disinfectant is removed. The storage unit is now disconnected and moved to its permanent location where the necessary service lines are attached.

DISCUSSION

The apparatus which has been described lends itself to the quantitative study of experimental respiratory infection in small laboratory animals. An aerosol of the infectious material can be prepared, the animals exposed to graded doses, and held in storage units for observation without danger to the operators of the equipment.

This apparatus has been used to produce respiratory infections of some of the most highly pathogenic bacteria and viruses. Respiratory challenge of immunized animals can be accomplished quantitatively. Antigens, either live or dead, can be administered in known doses for experimental immunization by the respiratory route. Animals that have been infected can be subjected to chemotherapeutic treatment inside the storage units. The pathogenesis of respiratory infections can be elucidated in animals which have been subjected to graded doses of the inciting agent. The epidemiology of respiratory infections can be studied in herds by introducing animals infected with this equipment.

A series of papers presenting the findings from continuing investigations in these fields is under preparation.

SUMMARY

An apparatus is described in which experimental air-borne infections may be quantitatively produced and studied. It consists of a series of interconnecting steel chambers containing an atomizer, exposure system, air sampling devices and auxiliary instruments and safety equipment.

A typical experimental operation is described. The various experimental studies to which the apparatus has been adapted, briefly mentioned here, will be presented in papers to follow.

STUDIES ON THE EXPERIMENTAL EPIDEMIOLOGY OF RESPIRATORY INFECTIONS

II. OBSERVATIONS ON THE BEHAVIOR OF AEROSOLS OF *STREPTOCOCCUS ZOOEPIDEMICUS*

I. L. SHECHMEISTER* AND L. J. GOLDBERG

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The recent information pertinent to air-borne infections has been reviewed at length by several investigators.[1–3] In the preceding article of this series, Leif and Krueger[4] described an apparatus which enabled quantitative evaluation of bacterial aerosols under varied controlled conditions and yielded reproducible results in certain investigations of host-parasite interaction. The relation of such factors as temperature, relative humidity, particle size, etc. to the quantative study of microbial aerosols was investigated and discussed by Rosebury and the staff of the laboratories of Camp Detrick, Frederick, Maryland,[5] and reference may be made to this publication for a comprehensive bibliography. The medical significance of aerosols is constantly attracting more attention, and the extension of information on this subject facilitates the study of the mechanism of air-borne disease. This was well exemplified by Bourdillon and his associates,[6] who carried out certain basic work on air hygiene and on transmission of air-borne infection in animals.

Previous reports in the literature on streptococcal aerosols dealt, for the most part, with the problems of control of streptococcal infections under a variety of environmental conditions. The pertinent bibliography was compiled by Coburn and Young,[7] who also pointed out the complexity and the importance of understanding the epidemiology of hemolytic streptococci.

We were interested in using group C streptococcus (*Streptococcus zooepidemicus*) for studies in experimental epidemiology of air-borne infections. These studies were designed (a) to evaluate the effect of atomization on streptococcal chain length and viability, (b) to determine the particle size of the viable

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1. Buchbinder, L. 1942, J.A.M.A. **118:** 718–730.
2. Personnel of Naval Laboratory Research Unit #1. 1943, War Med. **4:** 1–30.
3. Aerobiology, 1942, Publication No. 17 of the A.A.A.S., F. R. Moulton, Editor, Lancaster, Pa., The Science Press Printing Co.
4. Leif, W. R. and Krueger, A. P. 1950, J. Infect. Dis. **87:** 103–116.
5. Rosebury, T. and the Staff of the Laboratories of Camp Detrick, Md. 1947, Experimental Air-borne Infections, Baltimore, The Williams and Wilkins Co.
6. Medical Research Council, Special Report Series #262. 1948, Studies in Air Hygiene, London, His Majesty's Stationery Office.
7. Coburn, A. F. and Young, D. C. 1949, Epidemiology of Hemolytic Streptococcus, Baltimore, The Williams and Wilkins Co.

aerosol, (c) to quantitate the recovery of the aerosol from various parts of the apparatus, (d) to study the effect of prolonged atomization on the sampling of bacterial aerosol, (e) to determine the influence of suspending fluids on recovery of streptococcal aerosol and (f) to compare the intranasal and inhalation titrations of *Str. zooepidemicus* in mice. The apparatus described by Leif and Krueger[4] was used in all experiments. It is the purpose of this communication to present some of the results obtained in these investigations.

MATERIALS AND METHODS

Source of organisms.—Group C strains of β-hemolytic streptococci were obtained from Dr. L. Lancefield of the Rockefeller Foundation for Medical Research, New York City, New York, and from Dr. W. Henle, Virus Diagnostic Laboratory, Children's Hospital, Philadelphia, Pennsylvania. The strains were grouped by means of Lederle streptococcus serum.

Mediums.—Nutrient broth, tryptose phosphate infusion broth, serum tryptose phosphate infusion broth and 4.5% bovine blood agar were used routinely in this investigation. Nutrient broth consisted of 3 g of beef extract and 5 g of Bacto-peptone dissolved in 1000 ml of water, and the reaction was adjusted to pH 7.0. Tryptose phosphate infusion broth was prepared by infusing 10 g of beef heart in 1000 ml of distilled water at 56 C for 1 hour. The mixture was then heated to 80 C and held at that temperature for a few minutes to coagulate the proteins. It was then filtered, the volume made up to 1000 ml, and 29.5 g of tryptose phosphate added to the filtrate. Tryptose phosphate consists of 20 g of Bacto-tryptose, 2 g of Bacto-dextrose, 5 g of sodium chloride and 2.5 g of disodium phosphate. After the reaction was adjusted to pH 7.0, the medium was brought to a boil, filtered and autoclaved. Hereafter this medium will be designated as TPI broth. The designation "serum-TPI" was used when 10% (by volume) bovine serum was added to the above medium.

Preparation of cultures.—All stock cultures were transferred monthly on 5% bovine blood agar slants. The bacterial growth of one slant was washed off with 2 ml of TPI broth and added to 50 ml of the medium required for the experiment which, after static incubation for 8 to 9 hours at 37 C, constituted the first passage. The second passage was prepared by seeding 0.5 ml of the first passage per 10 ml of medium and incubating for 16 hours. This second serial passage was used in all experiments.

Production and sampling of aerosol.—Aerosols were produced in the "infection system" of the apparatus for the study of experimental airborne infections by means of a Wells atomizer delivering uniformly small particles at an approximate rate of 0.2 ml per minute. Prior to the determination of the droplet diameter and the exposure of mice to an aerosol, the bacterial suspension was atomized for 15 minutes to produce more uniform streptococcal chains in the suspension and in the resultant cloud. Mice were then exposed for 3 minutes, and the aerosol was simultaneously sampled for 2 minutes with special capillary impingers containing Sorensen's 0.1*M* phosphate buffer at pH 7.0 to determine its bacterial content.

Total and viable counts.—Total counts were made with a Petroff-Hauser chamber, according to the technique recommended by the manufacturer. In all cases at least 100 fields were counted for each determination. The bacterial concentration of the aerosol was determined after the impinger samples were concentrated by centrifugation at about 4500 r.p.m. for 20 minutes and the packed organisms resuspended in a known smaller volume of liquid.

Viable counts were made by spreading 0.1 ml of the appropriately diluted suspensions by means of a bent glass rod on the surface of 3 to 4 % bovine blood agar plates which were rotated manually on a turntable. The plates were then incubated at 37 C for 24 to 48 hours. The well defined zone of hemolysis facilitated the counting of colonies.

Slit sampling device.—Aerosols produced from dilute suspensions were sampled by means of a modified Bourdillon slit-sampling device[8,9] consisting of a jet set at a distance of 1 to 2 mm from an agar plate surface. The jet itself is a streamlined rectangular opening, 0.28 mm×1 cm, made from a piece of a thin walled $\frac{3}{8}$-inch stainless steel tubing. The agar plate was held on a platform which was revolved at 1 r.p.m. The detailed construction of the instrument, as well as its evaluation, are described elsewhere.[9] It was possible to sample at a rate of 1, $\frac{1}{2}$ or $\frac{1}{4}$ cubic foot of air per minute. The mean size

8. Bourdillon, R. B., Lidwell, O. M. and Thomas, S. C. 1941, J. Hyg. **41**: 197–224.
9. Goldberg, L. J. and Shechmeister, I. L. 1950, In press.

of particles impacted with 50% efficiency, using a single jet, is given by the relation:[10]

$$\frac{\rho D_{50}^2 V}{X} = 76 \quad (1)$$

where ρ = density of the particle which in this report is approximately equal to 1, D_{50} = diameter in microns of particle impacted with an efficiency of 50%, V = velocity of air jet in meters per second and X = slit width in mm. When D_{50} is arbitrarily set at 0.5μ, $V = 76 \times 0.28/0.25 = 85$ m/sec. Under these conditions the jet operates at about 50% of its maximum flow rate. Since, as stated below, the mean diameter of the droplet of *Str. zooepidemicus*, as it leaves the atomizer, is about 1.1μ according to May,[11] sampling under the above conditions should result in greater than 90 ± impaction efficiency. This was obtained experimentally.[9]

Experimental animals.—Three- to four-week-old mice were used in all animal tests. Our own pen-bred colony, showing no indication of any infectious disease during the past 5 years, served as the only source of these animals.

Titration of a particular culture in animals was accomplished by the intranasal instillation of 0.05 ml of the culture into each of 6 to 10 mice. Only mice receiving identical inoculums were kept in the same cage. All dead animals were autopsied for evidence of any pulmonary involvement, and the 50% mortality end points (LIN_{50}), expressed as viable organisms, were computed according to the method of Reed and Muench.[12] The amount of bacterial aerosol retained, and lethal to 50% of the exposed animals observed for a 10-day period, was symbolized as LRE_{50}.

Determination of the droplet diameter.—Goldberg's analyzer was used to determine the particle size distribution of the viable portion of the aerosol which was made up of medium, bacteria and particles of mixed content.[10] The analyzer consists of 3 identical jet-slide units arranged in series so as to form a multiple air filter. Each jet is a streamlined taper from a tube 1 inch in diameter down to a slit 0.06×0.7 cm placed 1 mm from a slide coated with a vaseline film to prevent the impinged particles from being blown off during a given sampling interval. Air passed through the particle size analyzer was further sampled by the capillary impinger, which estimated the amount of bacteria still present in the air effluent from the analyzer. It was shown that if the jet air velocity of the analyzer is varied, the mean particle size retained varied according to the following relation:[10]

$$\frac{\rho D_{50}^2 V}{X} = 48^* \quad (2)$$

where ρ = density of the particle which in this report is approximately equal to 1, D_{50} = diameter in microns of particle impacted with an efficiency of 50% using a filter consisting of 3 identical jet-slide units arranged in series, V = jet air velocity in meters per second and X = width of jet in mm.

The majority of the particles smaller than D_{50} for a given air flow through the analyzer were sampled by the capillary impinger, whereas most of the larger particles remained in the air filter.

The use of a radioactive tracer in cloud studies.—To aid in the evaluation of an aerosol, radioactive material was introduced into the atomizer fluid. The air samples taken from the aerosol were then more readily compared. We have found it convenient to use one microcurie of P^{32} per ml of atomized bacterial suspension.

Computation of percent recovery.—Percent recovery of bacterial aerosol was computed according to the method described by Rosebury et al.[8] This method yields the "nominal recovery," and is derived from the relationship between the number of organisms recovered from the cloud and the theoretical maximum number present in the cloud, in the manner indicated below:

(1) The theoretical maximum cloud concentration (i.e., the concentration per liter of cloud sprayed) =

$$\frac{\text{(volume of suspension sprayed) (concentration of suspension)}}{\text{(air flow, in liters per minute) (period of spraying, in minutes)}}$$

10. Goldberg, L. J. 1950, In press.
11. May, K. R. 1945, J. Sci. Instr. (London), **22**: 187–195.
12. Reed, L. J. and Muench, H. 1938, Am. J. Hyg. **27**: 493–497.

* Note that while equation (1) applies to a single jet, equation (2) is for the analyzer consisting of 3 jet-slide units.

(2) Concentration of cloud per liter, as recovered =

$$\frac{\text{(concentration per ml of sampler fluid) (volume of sampling fluid)}}{\text{(volume of cloud sampled, in liters)}}$$

(3) Nominal percent recovery = 100 × (2)/(1). The volume of bacterial suspension atomized per minute, the duration of spraying, the concentration of the bacterial suspension before and after atomization, as well as the assay of the aerosol samples for their viable bacterial content were determined for each experiment.

TABLE 1.—*Sample protocols illustrating breakup of chains of* Str. zooepidemicus *by atomization encountered in aerosol production.*

Number cells per chain	Example 1 Chains per 100 squares		Example 2 Chains per 100 squares	
	Before atomization	After atomization	Before atomization	After atomization
1	47	470	74	400
2	120	340	155	265
3	41	27	38	51
4	57	7	98	16
5	13	1	19	6
6	31	3	44	1
7	8		9	
8	24		15	
9	5		3	
10	7		5	
11	0		0	
12	5		2	
13	1			
14	3			
15	1			
16	4			
17	1			

TABLE 2.—*Breakup of chains of* Str. zooepidemicus, *as a function of pipetting, atomization and both pipetting and atomization.*

Number cells per chain	Chains per 100 squares			
	Before atomization		After atomization	
	No pipetting	Pipetting	No Pipetting	Pipetting
1	81	90	297	331
2	195	227	262	263
3	113	106	101	108
4	108	116	61	32
5	47	37	25	16
6	86	43	14	4
7	16	7	1	1
8	37	17	2	2
9	6	4	0	0
10	8	11	1	
12	8			
13	1			
14	5			
15	1			
16	5			

RESULTS AND DISCUSSION

The behavior of the bacterial suspension in the atomizer.—The principles of the operation of a Wells atomizer embody the impaction and refluxing of the bacterial suspension against the wall of the instrument. The effect of impaction upon a chain-forming organism, such as the streptococcus, and the corresponding viable count had to be ascertained before evaluating the behavior and characteristics of an aerosol. To this end, viable and total counts were made on bacterial suspensions of varied concentrations before and after subjecting them to atomization for different periods of time. In such an evaluation, it was important to determine the effect of agitation inherent in the routine dilutions of bacterial suspensions upon the chain length and survival of the streptococcus. A definite reduction in the chain length, as a result of the serial dilution of bacterial suspensions before and after atomization, is indicated in the results recorded in tables 1 and 2. However, this effect was greatly enhanced by the atomization process; pipetting resulted

TABLE 3.—*The effect of atomization on (a) the chain length, (b) the viable count and (c) the total count of* Str. zooepidemicus.

	Number of observations	Before atomization	After atomization	Percent increase in chains after atomization
Mean viable count per ml	7	6.9×10^8	1.3×10^9	83
Mean total count per ml	7	9.1×10^8	1.4×10^9	69
Mean number cells/chain	7	3.5 ± 0.26 $\sigma = \pm 0.82$	1.8 ± 0.17 $\sigma = \pm 0.42$	88

in 10 to 14% increase in the number of chains, compared with 88% increase encountered after atomization (table 3).

It was apparent that atomization for 15 minutes effectively broke up chains of any appreciable length, yielding mostly 1- or 2-cell aggregates. The observed when dilute suspensions were atomized. That this process was not lethal to the organisms was indicated by the respective total and viable counts before and after atomization. The mean streptococcal chain length observed in samples of 8 aerosols was 1.3 ± 0.2 cells,

TABLE 4.—*Total and viable counts of* Str. zooepidemicus *as a function of atomization time.*

	Number of observations	Before atomization	After atomization, minutes		
			5	15	45
Mean total count per ml	3	5.5×10^8	1.1×10^9	9.8×10^8	1.1×10^9
Mean viable count per ml	3	7.6×10^8			1.2×10^9

resultant average viable counts agreed well with the total counts and the increase noted subsequent to atomization (table 3). An average increase of 91% in viable count after atomization, demonstrated in 61 other experiments, compares very favorably with the computed value based on the difference in the number of cells per chain before and after atomization. Similar results were indicating an additional break-up during sampling. However, from table 4 it would appear that a similar chain break-up occurred after 5 to 45 minutes.

These results established the necessity of using the viable counts of bacterial suspensions determined after atomization in computing percent bacterial recovery. The mean chain length in bacterial suspensions after atomiza-

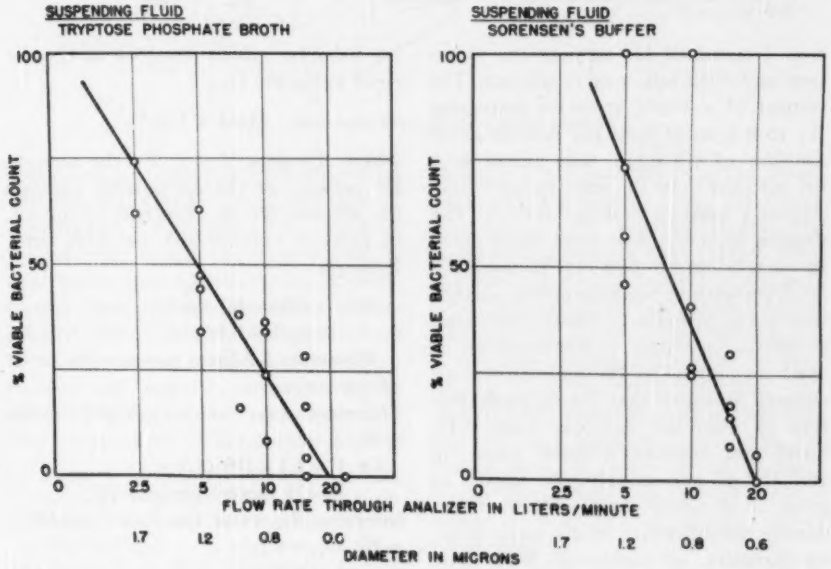

FIG. 1.—Cumulative particle size distribution of atomized *Str. zooepidemicus* suspended in a tryptose-phosphate broth and in Sorensen's buffer.

tion agreed well with the data presented below on the particle size of the bacterial aerosol. The above results confirmed the established uniformity of the aerosol and extended the reliability of the viable counts by defining the limits of the mean chain length responsible for colonial growth.

Determination of the particle size of the aerosol of Str. zooepidemicus.—The mean diameter of the aerosol particles was determined for suspensions atomized under the following conditions. The output of a Wells atomizer delivering 0.1 to 0.2 ml of fluid per minute at an air flow of 0.3 c.f.m. was mixed with dry air (less than 1% relative humidity) to give a total air flow of 1.0 c.f.m. The droplet diameter was then determined by correlating the flow rate through the analyzer with the corresponding particle size, using formula 2. These results are presented in figure 1. Examination of the lines plotted by the method of least squares indicates that the mean diameters of bacterial aerosols from TPI broth and Sorensen's buffer were 1.2 and 1.0 μ, respectively. To insure as great an accuracy as possible in determining the difference in the mean droplet diameter, all conditions were kept constant with the exception of the suspending fluid.

An example of data from a single typical experiment is presented in table 5. The viable recovery after the initial 15 minutes of atomization was assumed, for purposes of computation, to have changed linearly during the experiment, usually lasting 1 hour. This was considered in determining the corrected percent recovery. The following set of computations were used to correlate the flow rate through the analyzer with jet velocity, which resulted in the desired value for D_{50}.

TABLE 5.—*Determination of D_{50} when* Str. zooepidemicus *was atomized from TPI and serum and Sorensen's phosphate buffer.*

Impinger samples	Sampling rate, liters/min	Flow rate though analyzer, liters/min.	D_{50} microns	Viable count per ml of sampling fluid		Corrected† percent recovery
				(a) Recovered	(b)* Expected maximum	
			TPI and serum			
1–3	5.2	By-passed		1.06×10^8	1.06×10^8	100
4–6	5.2	5.2	1.2	0.35×10^8	1.02×10^8	34
7–9	5.2	10.0	0.84	0.08×10^8	0.99×10^8	8
10–12	5.2	15.0	0.70	0.04×10^8	0.96×10^8	4
13–15	5.2	By-passed		0.92×10^8	0.92×10^8	100
			Sorenson's phosphate buffer			
16–18	5.2	By-passed		7.9×10^4	7.9×10^4	100
19–21	5.2	5.2	1.2	5.0×10^4	6.8×10^4	73
22–24	5.2	10.0	0.84	1.5×10^4	5.7×10^4	27
25–27	5.2	15.0	0.70	0.07×10^4	4.6×10^4	18
28–30	5.2	By-passed		3.37×10^4	3.4×10^4	100

* Computed on the basis of linear decrease in the viable count.
† Ratio of (a) to (b).

$$(3)\quad A\times V=R$$

where A = area of slit, V = the average jet velocity of the air passing through the slit and R = the flow rate of the air, in units of volume flow per unit time. Example:

Slit: width = 0.7 cm
length = 0.06 cm

Flow rate: 5.2 liters per minute, or 87 ml per second

Therefore (av. velocity) $\times$ (0.7 $\times$ 0.06) = 87

Or $V=2.1\times10^3$ cm/sec
$V=21$ meters per second

Inserting the value for V into $D_{50}^2V/x = 48$, $D_{50}=1.2\mu$.

It is thought that a liquid drop formed in the atomizer subsequently

evaporates down to a core.[13] This core consists of all solids present in the orignal droplet; consequently, the greater the percent solids present in this droplet, the larger the final core size. This contributes to the difference in particle size when different suspending fluids containing different concentrations of solids are used.

A knowledge of the particle size distribution of the droplets is necessary in evaluating the effect of aerosol inhalation. One important factor in the depth of penetration of the particulate material into the respiratory tree is the size of the droplets.[14] The initial localization of the material in the respiratory tract is now taken into consideration in computing the over-all retention of the inhaled aerosol. Our results (fig. 1) show the narrow range of particle size distribution and suggest, because of the mean diameter of the aerosol particle, that localization of the greater part of the inhaled viable material occurred in the deep pulmonary tissue.

Recovery of bacterial aerosol from various parts of the infection apparatus.—It was possible to evaluate the role of the selected localized environmental conditions and performance of the apparatus on aerosol recovery by sampling at different locations in the infection system.

The possibility of a change in the viable aerosol concentration as a function of the secondary air supply and the distance of the sampler from the atomizer was investigated, using a radioactive tracer (P^{32}) introduced into the bacterial suspension immediately prior to atomization. Both agents were simultaneously recovered from the 3 sampling positions (ports) indicated in figure 2. A capillary impinger was used to sample the bacterial content of clouds produced from concentrated suspensions, and the slit sampling device was employed for the more dilute aerosols. Using this sampler, it was possible to assay as few as 5 organisms per 10 liters of air.

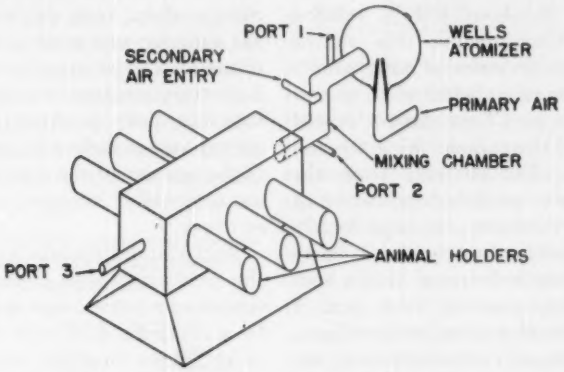

FIG. 2.—Schematic diagram of infection system.

Table 6 shows the recovery of the agents at the designated parts of the "infection system." Sampling from port

13. Phelps, S. 1942, Aerobiology, 133–137. Publication No. 17 of the A.A.A.S., F. R. Moulton, Editor, Lancaster, Pa., The Science Press Printing Co.

14. Hatch, T. F. 1942, Aerobiology, 102–105. Publication No. 17 of the A.A.A.S., F. R. Moulton, Editor, Lancaster, Pa., The Science Press Printing Co.

TABLE 6.—*Recovery of P^{32} and viable bacteria from the aerosol of* Str. zooepidemicus *and different parts of infection system.*

Exp. no.	Port	Capillary impinger			Slit sampler	
		Viable count per ml of atomized suspension	Percent recovery		Viable count per ml of atomized suspension	Percent recovery
			Bacterial	P^{32}		Bacterial
A	I	5×10^{8}	29	48	5×10^{3}	20
	II	5×10^{8}	25	57	5×10^{3}	
	III	5×10^{8}	3	45	5×10^{3}	21
B	I	8×10^{8}	23	39	8×10^{3}	8
	II	8×10^{8}	13	51	8×10^{3}	13
	III	8×10^{8}	4	50	8×10^{7}	8
C	I	1.2×10^{9}	17	63	1.2×10^{3}	
	II	1.2×10^{9}	12	63	1.2×10^{3}	10
	III	1.2×10^{9}	7	63	1.2×10^{3}	10

1 was representative of the initial atomizer output at about 100 % relative humidity. After mixing this aerosol with dry air (introduced tangentially into the "mixing chamber"), it was sampled from port 2 and port 3, located at the end of the cylinder with the animal holders. The recovery from the latter port was probably indicative of the effect of distance and other locally prevalent conditions on the cloud. There was a progressive decrease in the bacterial recovery starting with port 1 when a concentrated bacterial suspension was atomized at the indicated conditions of temperature and humidity. Similar data were obtained when the slit sampler was used with aerosols of diluted bacterial suspensions. However, a contrary situation was noted with the simultaneously recovered P^{32}; the observed values indicated that regardless of the position of the sampler, there was no appreciable change in the percent recovery.

Bacterial destruction, encountered in the production and collection of concentrated aerosols, was also manifested by a comparison of such viable counts as 8×10^{5} per ml of the impinger sample

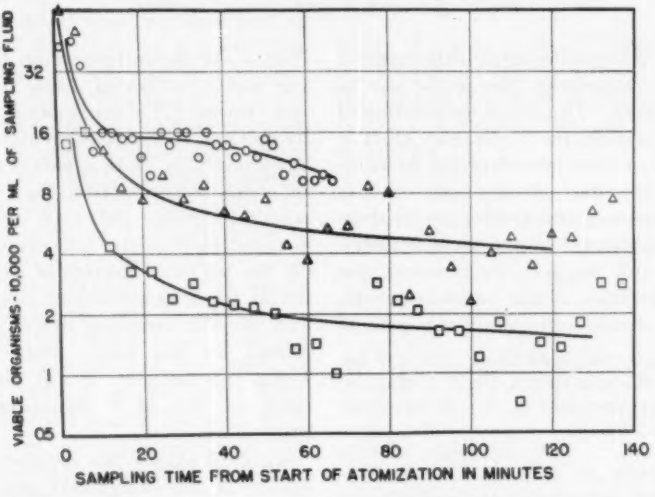

FIG. 3.—Successive sampling of aerosols of *Str. zooepidemicus* using a refluxing atomizer.

at port 3 with the total count of 5×10^6 per ml of the same sample. This decrease in the viable count of a dynamic cloud is particularly striking when compared with the consideraly higher recovery of P^{32}. Whether this is a direct result of such factors as the dilution of the aerosol, the difference in impingement on the walls of the apparatus or destructive drying of the bacterial load of the cloud by the secondary air supply is not definitely known.

Prolonged atomization of suspensions of Str. zooepidemicus.—Data presented in figure 3 show the need for an equilibration period of 8 to 15 minutes before reaching and maintaining a relatively constant aerosol concentration. The number of organisms sampled after the equilibration period fluctuated within rather narrow, well defined limits (table 8).

TABLE 7.—*The effect of prolonged atomization on the viable counts of different suspensions of* Str. zooepidemicus.

Time of atomization minutes	Viable counts $\times 10^5$/ml	
	Before atomization	After atomization
65	8.2	4.9
70	6.2	4.8
75	4.3	1.5
75	4.2	2.2
145	4.2	1.4

An illustrative series of 3 sets of 8 successive samples of aerosol, taken after equilibration, from 3 replicates of a bacterial suspension, yielded the following mean number $\times 10^5$ of organisms per ml of sampling fluid: (a) 1.1 ± 0.5, (b) 0.9 ± 0.12, (c) 0.9 ± 0.11, with respective standard errors of the mean of ± 0.17, ± 0.12 and ± 0.11. Other experiments substantiated this general trend, but samples in a given series occasionally showed a greater range of values.

It was previously demonstrated that atomization up to 45 minutes broke up streptococcal chains with a compensatory increase in viable count of the suspension (table 4). However, it is apparent from table 7 that after atomization for 65 to 145 minutes, the viable count was decreased about 2- to 3-fold.

In certain instances, it was necessary to maintain an aerosol from the same bacterial suspension over a long period of time. The importance of delineating the variation in bacterial recovery was evident when several groups of animals were exposed seriatim to the same aerosol. What effect such extensive agitation produced on the virulence of the resultant aerosol was not determined. All animal experiments were carried out after the established 15 minutes equilibration period and were completed well within 40 minutes from the start of atomization. Samples collected at the termination of the experiments were shown to be statistically indistinguishable from those obtained immediately after the equilibration period. During these experiments, the "infection system" temperature was maintained at 18 to 20 C and the relative humidity at 38 to 42%.

TABLE 8.—*Successive sampling of aerosols of* Str. zooepidemicus *after 15 minute equilibration period.*

Exp. no.	Mean organisms/ml sample $\times 10^5$ with standard deviation indicated	Standard error of the mean	Number of successive samples
A	1.3 ± 0.17	0.03	27
B	0.23 ± 0.12	0.02	27
C	0.55 ± 0.26	0.05	27

The effect of the "infection system" temperature on aerosol recovery was studied by atomizing aliquots of the same bacterial suspension and sampling the resultant aerosols at (a) to 10 to 12 C, (b) 18 to 19 C, (c) 32 to 37 C, keeping the relative humidity in the range of 35 to 38%. No significant difference in recovery was observed regardless of the temperature.

Influence of suspending fluids on recovery of streptococci from air-borne state.

—The percent recovery of a viable aerosol produced by atomizing bacteria suspended in different mediums is shown in table 9. For this phase of the investigation cultures were grown in TPI and serum, washed twice by centrifuging at about 2700 to 3000 r.p.m.

TABLE 9.—*Influence of suspending fluids on recovery of* Str. zooepidemicus *from air-borne state.*

Suspending medium	Percent recovery (mean of 6 experiments)
Dextrin-ascorbic acid	0.01
Tryptose-phosphate-infusion broth	3.5
Sorensen's phosphate buffer, pH 7.1	7.4
Gastric mucin, 6%	11.3
Tryptose-phosphate-infusion broth with 10% bovine serum	12.5

for 20 minutes and the sediment resuspended in the medium of choice. The presented data indicate that the percent aerosol recovery is a function of the suspending medium. Consequently, all subsequent bacterial suspensions were prepared in TPI and bovine serum. It was apparent that it is important to carefully standardize the suspending medium in comparing the percent aerosol recovery of organisms grown under different environmental conditions.

Comparative intranasal and inhalation titrations of Str. zooepidemicus *in mice.* —Inhalation titrations were carried out by exposing groups of 10 to 20 mice to progressively increasing aerosol concentrations for 3-minute periods and by determining the LRE_{50} according to the method of Reed and Muench.[12] The bacterial equivalent of LRE_{50} was computed from the following formula:

$$(4)\quad (T)\times(B)\times(W)\times(A)\times(R) = \text{number of inhaled and retained organisms}$$

where T = time of exposure of animals to aerosol; B = bacteria in ml of aerosol, determined directly from the impinger samples or computed for cloud in question by extrapolation from the known values; W = the average weight of exposed animals in grams; A = tidal air of the exposed animals. (On the basis of the oscilloscopic method devised by Guyton[15] for determination of the respiratory volume, the value of 1.25 ml per minute per gram of mouse was used in all calculations); R = retention of the inhaled bacteria in the respiratory tree, equal to 27% on the basis of the results presented by Goldberg and Leif.[16]

For example, if LRE_{50} aerosol concentration was 10 organisms per ml of air, then a 20-gram mouse exposed for 3 minutes retained $3\times10\times20\times1.25\times0.27 = 176$ organisms.

Intranasal instillation of serial dilutions of an aliquot of (a) bacterial suspensions and of (b) impinger aerosol samples allowed a comparison of the methods by determining the bacterial value of the LIN_{50} and LRE_{50}. Table 10 illustrates the relative response to dosage. It was observed that the number of organisms in the intranasal unit dose was much smaller than the number in the corresponding inhalation dose. The factors of retention of chains and their disaggregation complicate in opposite ways an explanation of this effect. At least the effectiveness of retention for

TABLE 10.—*Comparison of inhalation and intranasal LD_{50} values for* Str. zooepidemicus *in mice.*

Trial no.	Number of viable organisms per LD_{50}		
	Inhalation	Intranasal	
		Impinger sample	Bacterial suspension before atomization
22	500	100	40
24	400	60	40
26	400		100
31	350	120	150
33	350		

15. Guyton, A. C. 1947, Am. J. Physiol. **150:** 70–77.
16. Goldberg, L. J. and Leif, W. R. 1950, In press.

intranasal inoculation overcomes the probable decrease in LRE_{50} values, due to disaggregation, since the breakup of chains is not accompanied by a regular increase in the number of infective units. This is a difficult problem in the behavior of streptococcal aerosols, for it complicates the calculations necessary to produce clouds of a given concentration for exposure studies.

The determination of the bacterial equivalent of LRE_{50} value for a given organism is a prerequisite to evaluate its possible use in experimental epidemiology under controlled conditions. A high LRE_{50} may preclude the demonstration of indirect transmission of the agent. It is important to emphasize that the presented LRE_{50} values (table 10) resulted from exposure of mice for a short time to relatively concentrated aerosols. Whether or not prolonged exposure to a more dilute aerosol, possibly more indicative of the conditions encountered in nature, would result in similar values is not known. The determination of the relationship between the bacterial concentration of air and the duration of the exposure to the incidence of disease is possible under carefully controlled experimental conditions and is being investigated.

SUMMARY

Certain aspects of the behavior of aerosols of *Streptococcus zooepidemicus* were investigated prior to its use as an indicator organism in experimental epidemiology of air-borne infections.

Atomization of suspensions of the agent resulted in reduction of chains from 3.5 ± 0.26 to 1.8 ± 0.17 cells, and correspondingly, in an increase of about 90% in the viable and total counts of the suspensions.

Using Goldberg's particle size analyzer, the mean diameters of the droplet nuclei from TPI broth and Sorensen's buffer were 1.2 and 1.0 μ, respectively.

The change in viable bacterial concentration of the clouds was traced from the nozzle of the atomizer to the exhaust. In the case of the concentrated aerosols, a pronounced drop in viable aerosol was noted with the increase of the sampling distance from the atomizer nozzle. This was not the case with either the dilute aerosols or droplets containing P^{32}.

The viable count of bacterial suspensions decreased 2- to 3-fold after prolonged atomization of 65 to 145 minutes. The necessity of an equilibration period of about 15 minutes was demonstrated. Good replication of bacterial concentration in successive aerosol samples was indicated.

It was shown that the bacterial recovery was a function of the suspending medium, dextrin-ascorbic acid yielding the poorest and TPI and bovine serum the best recovery values.

A comparison of LIN_{50} and LRE_{50} values showed that the number of organisms in the intranasal unit was smaller than in the aerogenic unit.

These results are discussed in terms of their possible effect upon, or utilization in, the experimental epidemiology of air-borne infections.

STUDIES ON THE EXPERIMENTAL EPIDEMIOLOGY OF RESPIRATORY INFECTIONS

III. CERTAIN ASPECTS OF THE BEHAVIOR OF TYPE A INFLUENZA VIRUS AS AN AIR-BORNE CLOUD

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Previous reports in the literature on influenza virus have dealt with the successful infection of animals by the intranasal instillation of the virus from experimentally infected air.[1,2] This was followed by demonstrating air-borne infection of ferrets with the same agent.[3,4] The exposure of mice to influenza virus aerosol yielded a quantitative response to dosage of atomized virus.[5,6] Finally, contact transmission of the virus and the existence of an air-borne route of tion was established.[7–9] The further development of techniques to study air-borne infection has been reviewed in the first paper of this series, and reference for such information is therefore made to it.[10]

It is the purpose of this communication to present some of the results obtained in the study of the behavior of influenza virus aerosols. This investigation was conducted with the aid of the apparatus for the study of experimental respiratory infections described in detail by Leif and Krueger.[10] This instrument made possible the quantitative evaluation of the particle size and the viral content of the aerosols, and the determination of the relative efficiency of virus infection by the intranasal route and by inhalation of a virus aerosol.

MATERIALS AND METHODS

Virus.—The egg-adapted W. S. strain of type A influenza virus was used. This virus was obtained from Dr. M. D. Eaton, then direction of the California State Virus Laboratory. Three separate batches of 400 to 500 ml each of virus suspension were prepared by inoculation of 0.1 ml of 1×10^{-6} dilution of the preceding virus passage into the allantoic fluid of 11-day-old chick embryos. After incubation at 35 C for 40 to 44 hours, the eggs were chilled at 4 C for 4

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10. Leif, W. R. and Krueger, A. P. 1950, J. Infect. Dis. **87**: 103–116.

to 5 hours and the allantoic fluid harvested. The fluid was pooled and then subdivided into 20 ml aliquots. Each aliquot was tested for sterility, and the virus was stored in the dry ice chest (−72 C). Once a tube with virus was removed from the dry ice box, it was never returned.

Viral aerosols.—The viral aerosols were produced from dilutions of allantoic fluid virus suspensions prepared in 0.1 *M* Sorensen's phosphate buffer (pH 7.1) by means of a Wells atomizer[10] delivering uniformly small particles at the rate of approximately 0.2 ml per minute. Samples of these aerosols were collected with special capillary impingers using the Sorensen's phosphate buffer indicated above as the collecting medium, and were assayed for their viral content in the manner stated below.

Test animals.—Three- to four-week-old mice were used in all animal tests. Our own pen-bred colony, showing no indication of any infectious disease during the past 5 years, served as the only source of these animals. White Leghorn embryonated eggs were purchased from one local dealer and incubated to the desired age in our laboratory.

Assay of influenza virus.—Titrations were carried out in 11-day-old embryonated eggs and/or 21- to 23-day-old mice of approximately 10 g weight. Sorensen's 0.1 *M* phosphate buffer at pH 7.1 was used for all serial dilutions.

Five to six 11-day embryonated eggs were inoculated by the allantoic route with 0.1 ml of the test material, incubated at 35 C for 40 to 44 hours, subsequently chilled at 4 C for 4 to 5 hours, and then 0.5 ml of clear allantoic fluid was withdrawn from each egg for the determination of hemagglutinins.[11,12] Usually the results were "all-or-none" in nature. The maximum possible score for the red blood cell agglutination patterns was 4, while 0 represented the absence of any agglutination. Intermediate end points were determined by the pattern of the agglutinated erythrocytes. Fifty percent infectivity end points (ID_{50}) were calculated as suggested by Hirst's application of Reed and Muench's technique.[13,14]

When mice were used, 0.05 ml of the test material was instilled intranasally into each of 6 to 10 mice used for each tested virus dilution. Only the mice receiving an identical inoculum were confined to the same cage. All animals dying within 10 days after inoculation were autopsied for evidence of the degree of pulmonary involvement, and the 50% mortality end points (LIN_{50}) were computed according to the method of Reed and Muench.[14] Those surviving the 10-day holding period were sacrificed, the degree of lung consolidation noted[15] and the 50% maximum score end point (MS_{50}) computed.

The amount of viral aerosol inhaled and retained during a 3-minute exposure and lethal to 50% of the exposed animals, observed for a 10-day period, was symbolized as LRE_{50}.[16] In determination of LRE_{50}, groups of 6 to 12 mice were exposed seriatim for 3 minutes to 4 or 5 progressively increasing concentrations of the aerosol. Virus content of these aerosols was determined by titrating the impinger cloud samples intranasally in mice and/or eggs. Survivors of the inhalation exposure were sacrificed after the 10-day holding period, and inhalation LD_{50} was determined by the method of Reed and Muench. Prior to the exposure of mice to an aerosol or to a determination of the droplet diameter, every virus suspension was atomized for 15 minutes.

EXPERIMENTAL RESULTS

To determine the resistance of virus to atomization, aliquots of certain virus suspensions were titrated in eggs and in mice before and after the atomizing period. Representative results in table 1

TABLE 1.—*Titration of representative influenza virus suspensions in embryonated eggs and mice before and after atomization.*

Suspension no.	Concentration of suspension, titrated in			
	Eggs, virus units as ID_{50}/ml		Mice virus units as MS_{50}/ml	
	Before atomization	After atomization	Before atomization	After atomization
2	$10^{8.9}$	$10^{8.4}$	$10^{7.3}$	$10^{6.7}$
6	$10^{8.8}$	$10^{8.7}$	$10^{7.4}$	$10^{7.7}$
12	$10^{7.8}$	$10^{7.7}$	$10^{6.3}$	$10^{6.0}$
17	$10^{8.0}$	$10^{7.8}$		
20	$10^{7.8}$	$10^{8.5}$		
30	$10^{8.5}$	$10^{8.5}$	$10^{7.9}$	$10^{8.2}$

ID_{50} = quantity of virus infecting 50% of inoculated eggs.
MS_{50} = quantity of virus producing 50% of the maximum lung lesion score in mice.

11. Personnel of Naval Laboratory Research Unit #1, USNR, Univ. of Calif., Berkeley, 1943, U. S. Nav. Med. Bull. **41**: 114–128.
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indicate that the titers of these suspensions do not differ by more than may be expected from the variability inherent in the titration of viruses.[17] Since concentration of the suspension due to preferential fluid loss during atomization was not observed to be a factor with our refluxing atomizer, this agreement in titer was probably indicative of the resistance of this virus to atomization.

The percentage of virus recovery was computed with the aid of the following formulas:[18]

(1) The theoretical maximum cloud concentration (i.e., the concentration per liter of cloud sprayed) =

$$\frac{\text{(volume of suspension sprayed) (concentration of suspension)}}{\text{(air flow, in liters per minute) (period of spraying, in minutes)}}$$

(2) Concentration of cloud per liter, as recovered =

$$\frac{\text{(infective units per ml in sampler) (volume of sampling fluid)}}{\text{(volume of cloud sampled, in liters)}}$$

(3) Nominal percent recovery = $100 \times [(2)/(1)]$

This method yields the "nominal percent recovery value" and is derived from the relation between the number of infective units recovered from the cloud and the theoretical maximum number present in the cloud. The volume of the virus suspension atomized per minute, the duration of spraying, the titer of the virus suspension before and after atomization, as well as the assay of the aerosol samples for their virus content, were determined for each experiment. It is evident that the formulas above do not attempt to explain the discrepancy between the theoretical maximum number of infective units in the aerosol and the actual number of infective units.

The data presented in table 2 summarize the results of 26 experiments indicating the relation between the concentration of atomized virus suspension and the virus recovered from the resultant aerosol. Using mice for titration of these virus suspensions and the corresponding aerosols, the percentage of virus recovered was shown to be independent of the initial concentration of the atomized suspension. Within the range of the concentration studied, a constant nominal percent recovery was obtained, the observed differences being statistically not significant. Similarly, using the embryonated eggs for certain of these titrations, the average percent recovery, with its standard error, for 22 observations was 8.5 ± 1.7. Although there is no significant difference between the average percent recovery as determined using the two hosts above, variation within a particular experiment was occasionally pronounced.

The particle size of the aerosol, pro-

TABLE 2.—*Relation between the concentration of influenza virus in the atomized suspension and the resultant aerosols.*

Number of trials	Virus units as MS_{50}/ml of		Percent recovery
	Suspension	Aerosol	
8	$2.6 \pm 0.4 \times 10^7$	$3.0 \pm 0.9 \times 10^6$	6.4 ± 1.4
8	$1.9 \pm 0.1 \times 10^6$	$1.5 \pm 0.6 \times 10^5$	4.9 ± 1.6
10	$3.5 \pm 0.4 \times 10^5$	$7.2 \pm 0.7 \times 10^3$	8.5 ± 1.2

MS_{50} = quantity of virus producing 50% of the maximum lung lesion score in mice.

17. Stanley, W. M. 1946, Chem. Eng. News, **24**: 755.
18. Rosebury, T., Meiklejohn, G., Kingsland, L. C. and Boldt, M. H. 1947, J. Exper. Med. **85**: 65–76.

duced by atomization of influenza virus suspension, was determined by means of the "particle size analyzer," described in detail in the next paper of this series.[19] For this purpose 0.3 millicurie of radioactive tracer (P^{32}) was added to each 100 ml of 1:100 Sorensen's phosphate buffer dilution of the allantoic fluid virus suspension. The mass mean diameter of the droplets produced in these and other experiments reported at this time was less than 0.5 μ.

The effect of humidity on the percent recovery is indicated in table 3. The relative humidity of the virus aerosol was recorded during 19 separate experiments by determining the dew point of the cloud. It appears that a higher percent recovery of the viable aerosol was obtained at approximately 32 and 68% relative humidity, while at approximately 60% relative humidity the aerosol recovery was at a minimum. A similar phenomenon has been reported by Dunklin and Puck[20] in their study of the lethal effect of the relative humidity on air-borne bacteria.

TABLE 3.—*The relation between the relative humidity and the recovery of the influenza virus aerosol.*

Number of trials	Relative humidity, percent	Aerosol recovery, percent
1	30–34	13.0
3	41–45	6.2
5	50–54	5.2
4	58–60	0.9
6	66–70	10.4

The exposure of mice to aerosols of graded concentration allowed the expression of LRE_{50} in terms of LIN_{50} units. The virus equivalent of LRE_{50} was computed from the following formula:

$$(T)\times(C)\times(W)\times(A)\times(R)=LRE_{50}$$

(amount of inhaled and retained virus, expressed in LIN_{50} units)

where T = time of exposure of animals to aerosol; C = virus in ml of aerosol, determined directly from intranasal titrations in mice of the inpinger samples or computed for the cloud in question by extrapolation from the known values, and expressed in LIN_{50} units; W = the average weight of exposed animals, in grams; A = tidal air of the exposed animals (On the basis of the oscilloscopic method devised by Guyton[21] for determination of the respiratory volume, the value of 1.25 ml per minute per gram of mouse was used in all calculations.); and R = retention of the inhaled virus in the respiratory tree, equal to 27% on the basis of the results reported by Goldberg[22] in his determination of the retention of inhaled avirulent *Pasteurella pestis* tagged with P^{32}.

For example, if LRE_{50} aerosol concentration was 17 LIN_{50} units per liter of air, then an 8.3 mouse exposed for 3 minutes retained $3\times0.0019\times8.3\times1.25\times0.27=0.15$ LIN_{50} units. The data in table 4 indicate this relation between the inhalation and the intranasal LD_{50}.

TABLE 4.—*Response of mice to aerosols of influenza virus.*

Trial no.	Average mouse weight in grams*	Ml air inhaled in 3 min.	Cloud concentration LIN_{50} per liter	LIN_{50} equivalent of LRE_{50} unit
19	8.3	31	17	0.15
24	8.7	33	33	0.31
10	9.6	36	47	0.47
13	9.9	37	45	0.48

* Based on 20 mice.

DISCUSSION

For a series of clouds of graded concentration, there exists a reproducibility in the recovery of virus aerosol. A con-

19. Goldberg, L. J. 1950, J. Infect. Dis. **87:** 133–141.
20. Dunklin, E. W. and Puck, T. T. 1948, J. Exper. Med. **87:** 87–101.
21. Guyton, A. C. 1947, Am. J. Physiol. **150:** 70–77.
22. Goldberg, L. J. In press.

stant nominal recovery was obtained within the range of the investigated virus concentrations.

The mass mean diameter of influenza virus droplet was found to be about 0.5μ. Since the mass mean diameter of a distribution of droplets is greater than the mean diameter of the same droplet distribution, and since the diameter of the influenza virus is of the order of 0.1 μ,[23,24] there is a high probability that virus droplet nuclei of the indicated size range will produce a true pulmonary infection. "This does not, of course, preclude the possibility of absorption from the upper respiratory tract and subsequent transportation by body fluids to other areas."[25]

A comparison of the inhalatory method with the intranasal route of administration of India ink and radioactive chromic phosphate, as reported by the Personnel of the Naval Laboratory Research Unit #1 and, W. R. Lyons,[26] definitely established the superiority of the inhalatory method with respect to the distribution and penetration of both materials. In their opinion, with which the writer concurs, the intranasal instillation of fluids results in an uneven distribution of the material. The results presented in table 4 indicate that the W. S. strain of influenza virus used in these experiments was apparently characterized by greater infectivity when introduced as an aerosol than when instilled intranasally in the form of a suspension of the same cloud. This difference in the LRE_{50} and LIN_{50} values may be explained in terms of a disaggregation of infective units in the air-borne form, and a reaggregation either later on, in the cloud, or in the sampling fluid.

23. Friedewald, W. F. and Pickels, E. G. 1944, J. Exper. Med. **79**: 301–317.
24. Lauffer, M. A. and Stanley, W. M. 1944, J. Exper. Med. **80**: 531–548.
25. Hatch, T. F. 1942, Aerobiology, 102–105, Publication No. 17 of the American Association for the Advancement of Science, F. R. Moulton, Editor, Lancaster, Pa., The Science Press Printing Co.
26. Personnel of Naval Laboratory Research Unit #1, and Lyons, W. R. 1944, Am. J. M. Sc. **207**: 40–60.

SUMMARY

Certain aspects of the behavior of the aerosol of the W. S. strain of type A influenza virus were investigated.

The mechanism of production of the virus aerosol did not affect the titer of the virus as measured in mice. The percent recovery of the virus aerosol was independent of the initial concentration of the atomized suspension.

The effect of the relative humidity on the recovery of the aerosol was investigated. High aerosol recovery was obtained in the extreme ranges of 32 and 68% relative humidity, while a minimum recovery was obtained at 60% relative humidity.

The mass mean diameter of the influenza virus aerosol was determined to be less than 0.5 μ.

This influenza virus is apparently characterized by greater infectivity when introduced by the air-borne rather than by the intranasal route.

STUDIES ON THE EXPERIMENTAL EPIDEMIOLOGY OF RESPIRATORY INFECTIONS

IV. A PARTICLE SIZE ANALYZER APPLIED TO THE MEASUREMENT OF VIABLE AIR-BORNE BACTERIA

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The particle size distribution of viable air-borne bacteria is an important factor in the study of a bacterial cloud. Several techniques are used to determine the particle size distribution in aerosols, but they are not readily adaptable to the analysis of a viable component. The cascade impactor,[1,2] which was designed to divide a particulate cloud into several known particle size ranges, can be used to analyze a viable component since the bacterial recovery from any stage is directly correlated with a known size range. A more precise technique for analyzing bacterial clouds with a small particle size range comparable with the distribution on a single impactor stage has been developed and is described in detail below.

When a thin, streamlined, rectangular jet is set at 90 degrees to a sticky surface and a suitable particulate cloud is sampled, almost all the particles that impinge* on the surface lie in an area approximately the same size as the jet opening.[3] The impinging of particles consequently occurs during the time interval:

$$\tau \cong \frac{x/2}{V}$$

where x is the width of the slit or air-jet and V the jet stream velocity. The average stream displacement for impaction to occur is approximately x/4, assuming a uniform particle distribution in the bifurcated air stream, i.e., half the bifurcated stream width. The following formula[4] gives the distance a particle will be displaced during a time interval, t, if its motion is in accord with Stokes' Law:[4]

$$(1) \quad S = \frac{mVD_{50}}{12\mu A}\left[1 - \exp\left(-\frac{24\mu At}{mD_{50}}\right)\right]$$

where m = mass of sphere, V = initial velocity, D_{50} = diameter of sphere, A = cross-sectional area, and μ = viscosity of air.

Inserting the values $t \cong \tau$, $S \cong x/4$ yields the following formula:

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The opinions and assertions contained in this report are the private ones of the writer and are not to be construed as official or reflecting the views of the Navy Department or the naval service at large. (From Article 1252, U. S. Navy Regulations 1948.)

1. May, K. R. 1945, J. Sci. Inst. (London) **22**: 187–195.
2. Rosebury, T. 1947, Experimental Air-Borne Infections, The Williams and Wilkins Co., Baltimore, p. 57.

* The word "impinge" is used with reference to the process of adhesive impaction, whereas "impaction" is used only in the sense of striking against, still leaving the question of adhesion to the surface unanswered.

3. Bourdillon, R. B., Lidwell, O. M. and Thomas, J. C. 1941, J. Hyg. **41**: 197–224.
4. Dalla Valle, J. M. 1948, Micrometrics, New York, Pitman Publishing Co.

$$(2)\quad \frac{x}{4}=\frac{mVD_{50}}{12\mu A}\left[1-\exp\left(-\frac{12\mu Ax}{mVD_{50}}\right)\right].$$

According to formula (2), an equivalent percentage impaction should occur for all values of the variables satisfying the equation

$$\frac{mVD_{50}}{\mu Ax}=\text{constant}.$$

For a sphere, this expression becomes

$$(3)\quad \frac{\rho D_{50}^2V}{\mu x}=\text{constant}$$

since

$$A=\frac{\pi D_{50}^2}{4}$$

and

$$\frac{\rho\pi D_{50}^3}{6}=m.$$

If the air jet does not spread before impacting against a surface, no appreciable loss in filtering would be expected. This has been checked experimentally and found to be true, indicating that the distance from the jet to the surface is not a critical factor.[3,5]

If the air jet velocity varied by a factor of 4, the mean particle size impacted on the surface should vary by a factor of 2 (formula 3). This relationship suggests the use of a jet surface unit as a continuously variable particle filter.

For a thin, streamlined, rectangular jet impactor, the following experimental relationship was obtained by May:[1]

$$(5)\quad \frac{\rho D_{50}^2V}{x}=76$$

where x is the width of the jet slit in mm, V the velocity of the air jet in meters/sec., and D_{50} is the mean equivalent spherical diameter in μ of particles, 50% of which are impacted onto the slide under the conditions specified.

In the case of a capillary jet, one may derive formula (6):

$$(6)\quad \frac{\rho D_{50}^2V}{x}=38$$

where x = capillary diameter in mm. Since, for a rectangular slit where x is the width, h the length, and y the thickness of the effluent air stream, the following relation holds:

$$2yh=xh \quad \text{or} \quad y=x/2.$$

Then, for a capillary jet with x representing the capillary diameter, this expression becomes:

$$\frac{\pi x^2}{4}=\pi xy \quad \text{or} \quad y=x/4.$$

Therefore, formula (5) is transformed to formula (6). A capillary jet of diameter x operated with the same air velocity as a slit, width x, should impact particles of 0.7 the size of those filtered out by the slit. A preliminary check of formula (6) has been made experimentally, and the correction to the value of 38 was found to be applicable.

A baffle plate on one side of the rectangular jet alters the air-flow in such a manner as to make the entire air-flow go to one side rather than divide evenly. In this case one would expect formula (5) to become:

$$(7)\quad \frac{\rho D_{50}^2V}{x}=152$$

since the thickness of the effluent air stream is doubled. This jet form has been found useful for sampling large particle sizes.

It is desirable for a filter to reject or retain particles of a given size. For this purpose the use of a number of jets in series is suggested. The cumulative impaction efficiency of n identical jets,

5. Goldberg, L. J. and Shechmeister, I. L. 1950, In press.

each with a fractional impaction efficiency (f) for particles of a given size, is shown by the following formula:

$$(8)\quad I_n = \sum_{m=1}^{n} f(1-f)^{m-1} = 1-(1-f)^n$$

where f = fractional impaction efficiency of a single jet unit, m = summation index, and n = number of filter units. The value I_n approaches 1 for all values of f between 0 and 1, providing n is sufficiently large. Two or three jets in series are normally used because a greater number is usually not practical. A graphical evaluation of the use of 1, 2 and 3 jets in series is presented in figures 1 and 2. In the case of three identical jets in series, formula (5) becomes:

$$(9)\quad \frac{\rho D_{88}^2 V}{x} = 76 \quad \text{or} \quad \frac{\rho D_{50}^2 V}{x} = 48$$

because of the change in I_n (formula 8).[1]

METHODS AND MATERIALS

An apparatus similar in over-all design to that described by May[1] was constructed. The chief modification was the substitution of identical jets (0.6 mm×7.0 mm, set 1.0 mm from the slide surface) in place of the various widths employed by May in his cascade impactor.

The apparatus developed by Leif and Krueger[6] for generating and mixing aerosols was used.

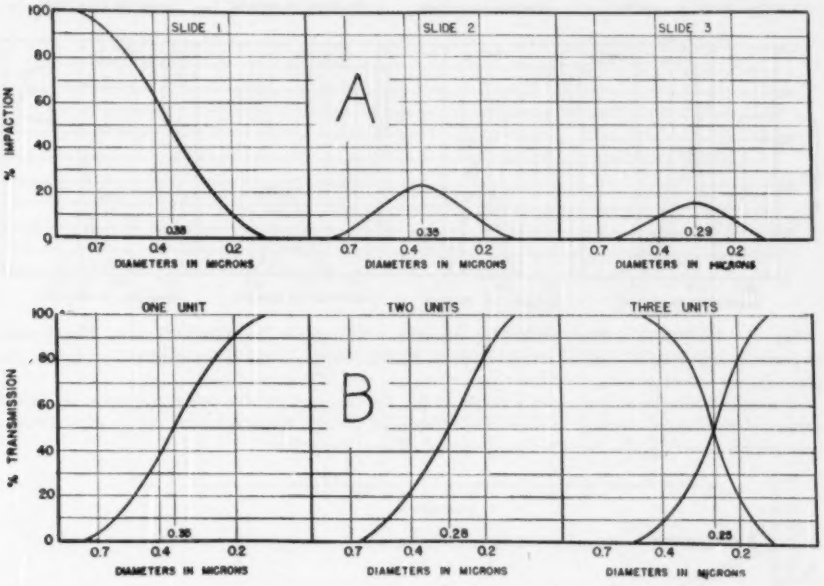

FIG. 1.—Analysis of a cloud containing particles of uniform size distribution. A. The theoretical particle size distribution on slides exposed simultaneously to three jets in series. B. Filtering characteristics of series jets.

Figure 3 is a schematic diagram of the system as used for this study, the operation of which is as follows:

The resistor valve, A, is adjusted so that its air-flow impedance is equal to that of the particle size analyzer. The air flow through the resistor valve, A, may be set at any desired value by means of the rotometer, R. The air flow through valve B is adjusted to maintain the air flow through the infection chamber at 1 c.f.m.

In order to determine the number of viable air-borne bacteria present in the infection cham-

6. Leif, W. R. and Krueger, A. P. 1950, J. Infect. Dis. **87**: 103–116.

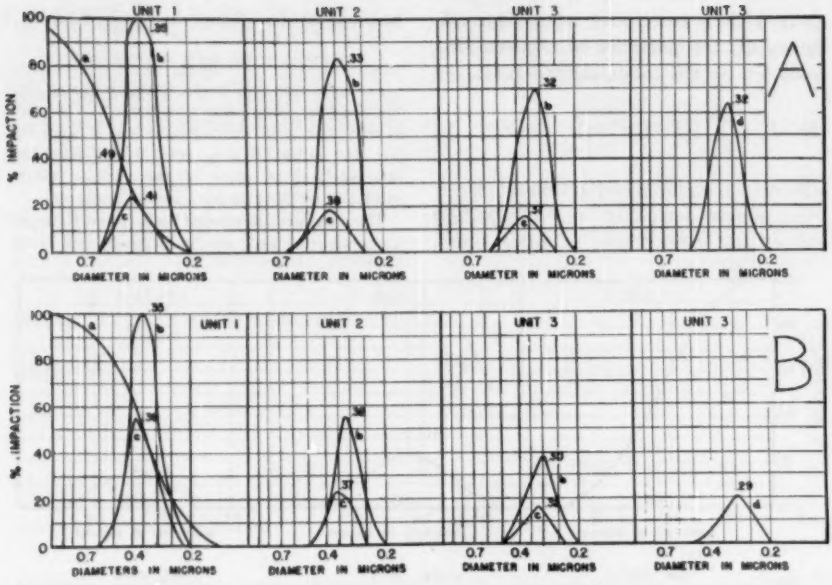

FIG. 2.—Analysis of a cloud containing particles with a given size distribution. A. Distribution of particles impacted at a given jet air velocity. B. Distribution of particles impacted at double the given jet air velocity.

a = assumed filtering efficiency of a single air jet; b = assumed particle size distribution of aerosol presented to filtering element; c = computed impinged particle distribution on sticky slide surface; d = computed particle size distribution of aerosol effluent from filter.

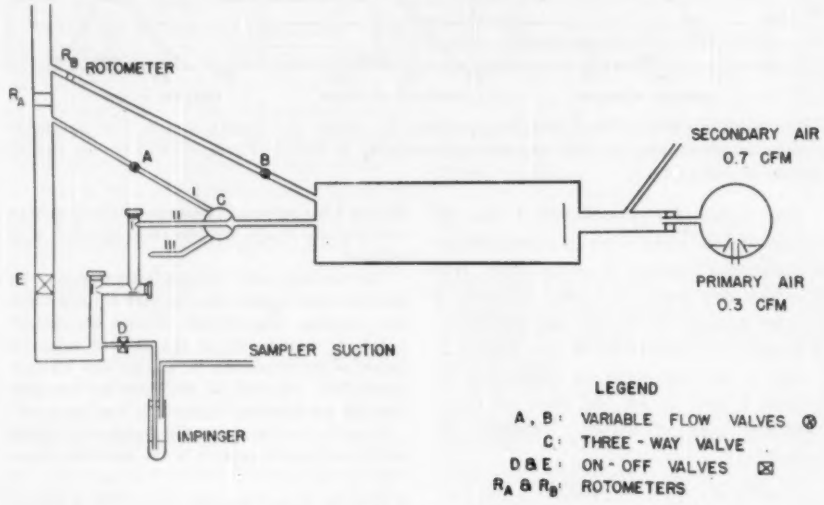

FIG. 3.—Schematic infection system.

ber, an air sample was taken by attaching a capillary impinger (I) operated at a sampling rate of 5 liters/min. to valve C in position III. After this, valve C was placed in position I. The air flow through the resistor valve A was set at a desired value, and valve B was adjusted to maintain the total air flow through the infection chamber at 1 c.f.m. A new impinger tube was attached at D. To obtain a sample, valve C was placed in position II and the impinger sampler suction turned on simultaneously. After sampling for the desired time, valve C was returned to position I and valves D and E were closed. This procedure permitted access to the particle size analyzer. The impinger samples were subsequently assayed for their viable bacterial content.

The viable bacterial counts were plotted as a function of particle size by means of formulas (5) or (9). These correlate jet velocity with mean particle diameter. The resulting graph represents the cumulative particle size distribution present in the cloud.

If the number of viable bacteria present in the cloud is extremely low, a direct sampling technique, such as a slit sampler,[3] may be used, thus eliminating the impinger sampler.

Pasteurella pestis strain A-1122 was grown in heart infusion broth (Difco) according to the procedure described by Goldberg and Shechmeister.[5] *Streptococcus zooepidemicus* was grown in tryptose phosphate infusion broth (Difco) plus 5% bovine serum.[5] In some cases the cells of *Past. pestis* were tagged with P^{32}, while only tagged *Str. zooepidemicus* was used. Aerosols were produced by atomization of washed cells suspended in the diluents described below.

Determination of particle size.—Analyzer slides for microscopic observation were prepared as follows: A thin coating of an Amine Alkyd solution A (The California Ink Co. Inc., Berkeley, Calif.) was spread on the slides and allowed to dry to tackiness. The slide was then inserted into the analyzer, and a short sample, at a desired flow rate, was taken. The slide was removed immediately and an additional droplet of the above solution placed over the deposit. A cover slip was placed on the liquid droplet and gently touched to eliminate entrapped air. Under oil immersion several hundred particles were counted and their sizes measured with a calibrated ocular micrometer.

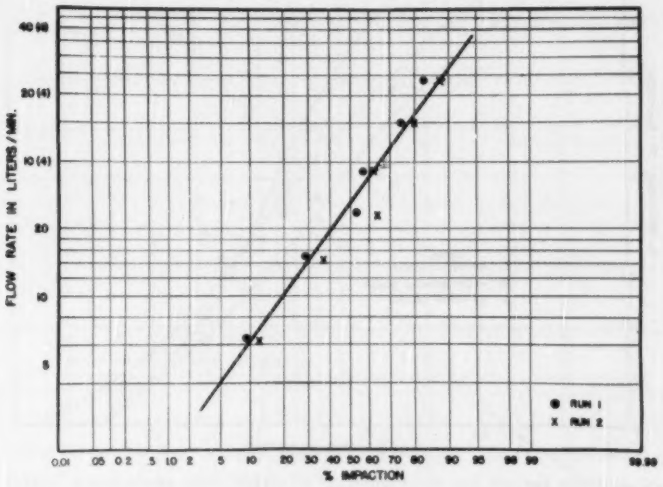

FIG. 4.—Experimental evaluation of D^2V = constant.

RESULTS

1. Radioactive solutions of 1% and 8% dextrin plus methylene blue dye were atomized and analyzed by means of the particle size analyzer. Since the size of the droplet nucleus is proportional to the cube root of the percentage of dissolved solids, the mean diameters of the particles of the aerosols should differ by a factor of 2. The data as

summarized in figure 4 indicate an excellent check of the formula:

$$\frac{\rho D_{50}^2 V}{x} = \text{constant.}$$

In plotting the values for the 8% solution, the flow rates used were multiplied by a factor of 4 and the results combined with those obtained using a 1% solution (fig. 4), which should result in a linear plot. This was obtained experimentally.

Slides exposed under various jet-air velocities to the second jet (0.6 mm × 7.0 mm set at a distance of 1.0 mm from the slide) in the particle size analyzer, consisting of 3 identical jets in series, were examined microscopically. The results are summarized in table 1.

The difference between the observed and calculated values for the particle size lies within the limits of experimental error. The particle size given in table 1 can most suitably be called the mode of the observed distribution, since many of the smaller particles are not counted in a microscopic check of this nature.

If a cloud of particles with a mean diameter of 1μ was sampled at a velocity of 40 meters/sec. through a jet 0.6 mm × 7.0 mm, set 1 mm from a surface of vaseline, half of the cloud sample would be retained on the surface while the other half would be carried away by the effluent air from the jet. Inserting the values

$$m = \frac{\rho \pi D_{50}^2}{6}, \qquad A = \frac{\pi D_{50}^2}{4}, \qquad \sigma \doteq 1,$$

$\mu = 1.8 \times 10^{-4}$ poise

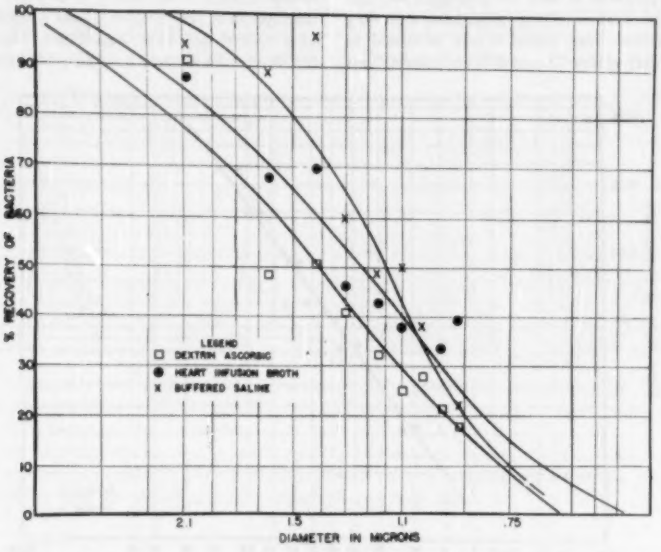

FIG. 5.—Cumulative particle size distribution of P^{32}-tagged *Past. pestis* (strain A1122) atomized from three suspending fluids.

TABLE 1.—*Particle size of dextrin impinged as a function of air jet velocity.*

Air jet velocity in meters/sec.	Particle size in μ
20	1.3
40	1.0
80	0.5

in formula (2), one obtains S = 0.12 mm. The average distance a particle is displaced before being impinged on the vaseline surface was assumed to be approximately x/4 where x is the width of the jet. This value is 0.15 mm which is in good agreement with the value derived from formula (2), and indicates that the theoretical formula correlated well with the experimental observations.

2. Aerosols of *Past. pestis* tagged with radiophosphorus were generated from suspensions prepared in the following fluids: heart infusion broth (Difco), dextrin plus ascorbic acid (2% dextrin, 1.5% ascorbic acid, 10 cc Sorenson's buffer), and buffered physiological saline (pH 7.4). A particle size analyzer with a single filtering stage was used. The results, graphically presented in figure 5, show that the suspending fluid affects the mean particle size. The mean particle size from all fluids obtained was in approximate agreement with the mean cell size determined by microscopic observation. It was calculated that airborne bacteria represented only a fraction of 1% of all the particles present in the cloud.

3. Suspensions of 10^8 and 10^{10} nonradioactive organisms per ml were prepared and atomized from cultures of *Past. pestis* which were grown, concentrated and then diluted in heart infusion broth. The particle size analyzer was used to determine the mean particle

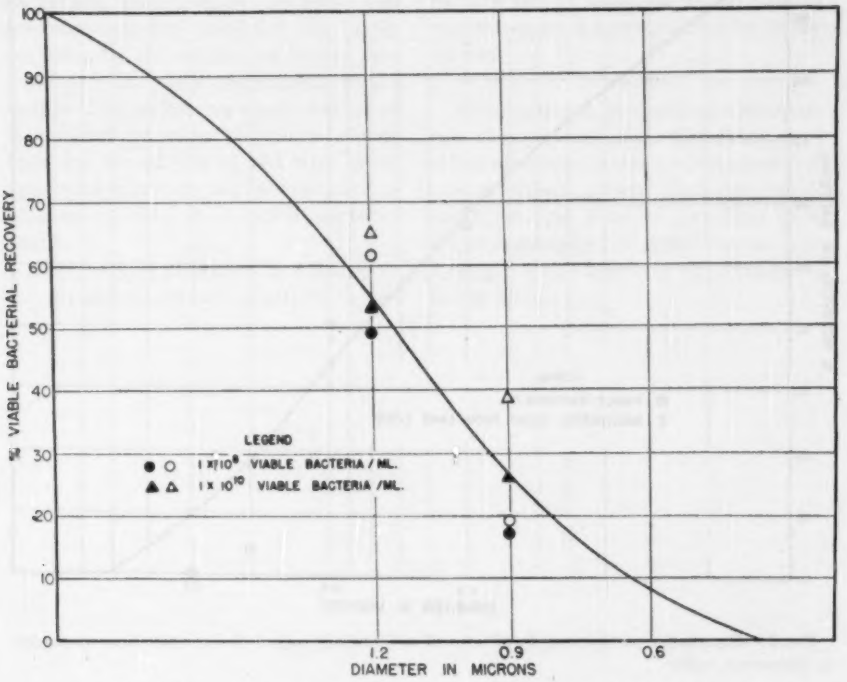

FIG. 6.—Cumulative particle size distribution of *Past. pestis* (strain 139L) atomized from heart infusion broth: the effect of a hundredfold decrease in the viable bacterial count.

size of each cloud (fig. 6). Although the mean particle size did not differ for a suspension derived from the same culture, differences were noted between cultures grown on subsequent days.

4. The cumulative particle size distribution of a cloud of *Str. zooepidemicus* tagged with radioactive phosphorous and suspended in Sorenson's buffer (pH 7.1), was also determined. The mean particle size was computed to be 1μ. The data are graphically represented in figure 7.

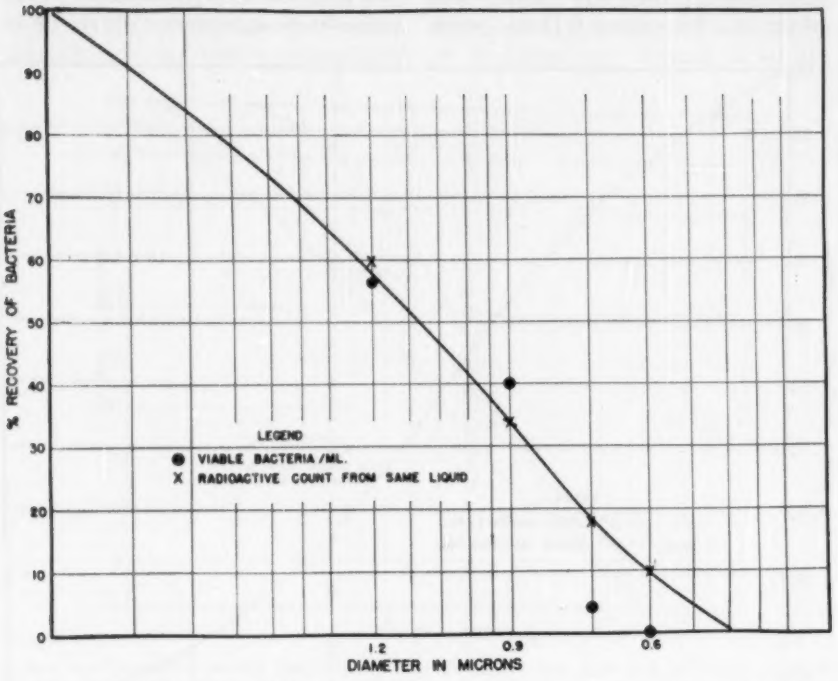

FIG. 7.—Cumulative particle size distribution of radioactively tagged *Str. zooepidemicus* suspended in Sorensen's buffer.

DISCUSSION

A viable bacterial assay may be made of the deposit on the slide exposed to the second or third jets of the particle size analyzer (figs. 1 and 2). This method of sampling lends itself to a direct analysis of the particle size distribution of the viable bacterial content of the cloud. However, one must consider the effect of the variation in jet velocity on the recovered viable bacteria.

The width of the jet to be used in a given application is dependent upon the range of particle sizes to be analyzed; the length of the jet to be used is dependent upon the sampling rates to be employed. Consequently, these factors must be considered in the use of jets in a particle size analyzer. For large particles approximately 10 μ in diameter, a jet with a baffle should be used to keep all the flow in one direction after impaction so that losses in the instrument may be avoided. For small particles, ap-

proximately 1 μ in diameter, a baffle is not desired.

It should be noted that in the technique of evaluating the cumulative aerosol distribution, an impinger sample was used which disrupted large particles containing many viable bacteria, and one consequently weighted the larger particles by an additional factor proportional to their contained viable counts. This weighting would not occur if a direct sampling technique, developed by Bourdillon et al,[3] were used, since each clump would be evaluated as only one colony in a viable bacterial count.

The method presented in this report for obtaining aerosol diameter is not immediately applicable to general usage where a wide range of particle sizes is to be expected. It is of value mainly as a laboratory tool to compare, for example, one set of atomizing conditions with another in which the expected change in particle size is small, as in the case of various suspending fluids used for atomization.

SUMMARY

A technique is outlined for determining the particle size distribution of viable air-borne bacteria when present in concentrations greater than one viable bacterium per liter of air. This technique is described in detail for particles having a mean diameter in the range of 0.6 to 2.0 μ.

IN VITRO STUDIES OF BACTERIAL RESISTANCE TO CHLORAMPHENICOL (CHLOROMYCETIN)

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The tendency of bacterial populations to alter in resistance to antibiotics raises intriguing questions for the geneticist and necessitates watchfulness by the clinician. Abraham (1941) adapted a strain of *Staphylococcus aureus* to penicillin in vitro. In the course of 16 weeks of frequent subculturing, its resistance increased 1000-fold. Rammelkamp (1942), working with two strains of *Staph. aureus*, obtained a 64-fold increase in resistance during 8 weeks of subculturing. Since then a host of similar experiments has been performed with various antibiotics.

Demerec (1949) described two stepwise patterns of in vitro emergent resistance for *Staph. aureus* and *Escherichia coli*. One pattern is characterized by slight increases of resistance in successive transfers in the presence of the antibiotic, so that high resistance is attained only by numerous transfers; this gradual pattern reflects the fact that variation in degree of resistance among the resistant cells of the same step is slight. The other pattern differs in that the variation between first-step resistant cells is very great, some of them being only slightly more resistant than the original strain, whereas other cells are extremely resistant, so that high resistance may be attained in a single transfer. The first pattern is characteristic of penicillin and apparently of aureomycin; the second, of streptomycin. It is noteworthy that these patterns are reflected in clinical experience. The emergence of in vivo resistance to streptomycin has become a grave obstacle to its prolonged administration, but thus far this problem is apparently less serious with penicillin and aureomycin.

The present studies were undertaken to ascertain the rate and extent of expected change in the resistance of some bacterial populations during and after cultivation in chloramphenicol-containing broth. The recent statement by McLean et al (1949) represented a preliminary report of these studies. Seven cultures, chosen as representatives of high and low resistance, were used to study changes in acquired resistance, loss of resistance, and chloramphenicol concentration during incubation.

EXPERIMENTAL METHODS AND RESULTS

Transfers in broth plus chloramphenicol (R series)

Stock solutions of chloramphenicol (Chloromycetin†) in Bacto Brain Heart Infusion broth plus 10% unpreserved horse serum were prepared in the following concentrations: 1, 5, 10, 15, 20, 25, 30, 40, 50, 75, 100, 250, 500, 750, 1000, 1250, 1500, 1750, 2000, 2250, 2500, 2750, 3000, 3250, 3500, 3750, and 4000 μg chloramphenicol per ml. These stock solutions were sterilized by Seitz filtration and tubed (2 ml/Wasserman tube).

A series of these tubes of broth containing chloramphenicol was then inoculated with one of each of the following cultures of bacteria:

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The authors wish to express their appreciation to three of their associates in the Parke Davis laboratories: to Mrs. M. Galbraith, for chloramphenicol assays; to Miss I. Ferguson, for cultural examination and lyophilization of *Esch. coli*, and to Dr. W. P. Iverson, for assistance with the manuscript.

† Parke, Davis & Co.'s trademark for chloramphenicol.

Klebsiella pneumoniae P-D #04544, *Aerobacter aerogenes* P-D #0126, *Salmonella typhosa* Keifer Patient #8, *Esch. coli* P-D #01495, *Proteus* sp. P-D #04736, *Pseudomonas aeruginosa* P-D #01925, and *Alcaligenes metalcaligenes* P-D urine isolate.

The initial inoculation into the chloramphenicol-containing medium was made from Bacto Brain Heart Infusion broth cultures that had been incubated for 18 to 24 hours at 37 C. The amount of inoculum for each transfer was 0.1 ml. After inoculation, the tubes were incubated for 72 hours at 37 C. Each subsequent transfer was made, from the tube with the highest concentration of chloramphenicol showing growth, into tubes of broth containing higher concentrations of the antibiotic. Each culture was transferred 20 times in this manner.

The results of this experiment are depicted as the R series in figure 1, and susceptibility after the 12th and 20th transfers is expressed in the 3rd and 8th columns of figures in table 1 in terms of ratio to original susceptibility. All of the organisms increased in resistance to chloramphenicol, their rates of increase being gradual. For *Proteus*, *Ps. aeruginosa*, and *Alc. metalcaligenes*, whose initial resistance was relatively great (inhibited by not less than 500 μg chloramphenicol per ml broth), the extent of increase could not be measured because the greatest concentration tested was not inhibitory, being limited by the solubility of the antibiotic.

S. typhosa and *Esch. coli*, whose initial resistance was much lower (inhibited by 10 μg per ml), increased in resistance 50 and 125 times, respectively, during 20 transfers. *K. pneumoniae* and *A. aerogenes*, which were initially the least resistant of the species studied (inhibited by 5 μg per ml), exhibited somewhat lower rates of increasing resistance: 5 and 10 times, respectively, during the first 12 transfers, and 10 and 50 times, respectively, over the entire 20 transfers.

Subsequent transfers in chloramphenicol-free broth (T series)

After the 12th (R12) and 20th (R20) transfers in broth containing chloramphenicol, 0.1 ml portions of these cultures were also removed to chloramphenicol-free broth and 0.1 ml portions were transferred daily (T1–T20). These chloramphenicol-free cultures were tested at intervals in chloramphenicol-containing broth to determine the extent to which their acquired resistance may have been lost.

The results of this experiment are depicted as the T series (from R12) and T′ series (from R20) in figure 1. Susceptibility after the 12th and 20th transfers is expressed in table 1 (4th and 5th and 9th and 10th columns of figures, respectively) in terms of ratio to original susceptibility. Decreased resistance after

TABLE 1.—*Changes in susceptibility to chloramphenicol after cultivation in the presence of the antibiotic (R) and after subsequent cultivation in its absence (T).*

Organism	Original minimum inhibitory concentration	Number of transfers				Decreased resistance	Number of transfers				Decreased resistance
	R1	R1 → R12 → T12 → T20				R12→T20	R1 → R20 → T′12 → T′20				R20→T′20
	μg/ml	Ratio					Ratio				
K. pneumoniae	5	1	5	1	1	5.0X	1	10	3	2	5.0X
A. aerogenes	5	1	10	4	3	3.3X	1	50	8	4	12.5X
S. typhosa	10	1	25	3	2	12.5X	1	50	25	7.5	6.6X
Esch. coli	10	1	50	25	7.5	6.6X	1	125	75	50	2.5X
Proteus sp.	500	1	>8	>8	5	>1.6X	1	>8*	>8	4	>2.0X
Ps. aeruginosa	1250	1	>3	3	2.6	>1.2X	1	>3	>3	2.8	>1.1X
Alc. metalcaligenes	1250	1	>3	3	2.8	>1.1X	1	>3	>3	3.2	>1.0X

* Transfers R18 and R19 failed to grow. When subcultures from these tubes in chloramphenicol-free broth also failed to grow, transfer R17 was subcultured in chloramphenicol-free broth. This subculture grew and retained its resistance in subsequent R transfers.

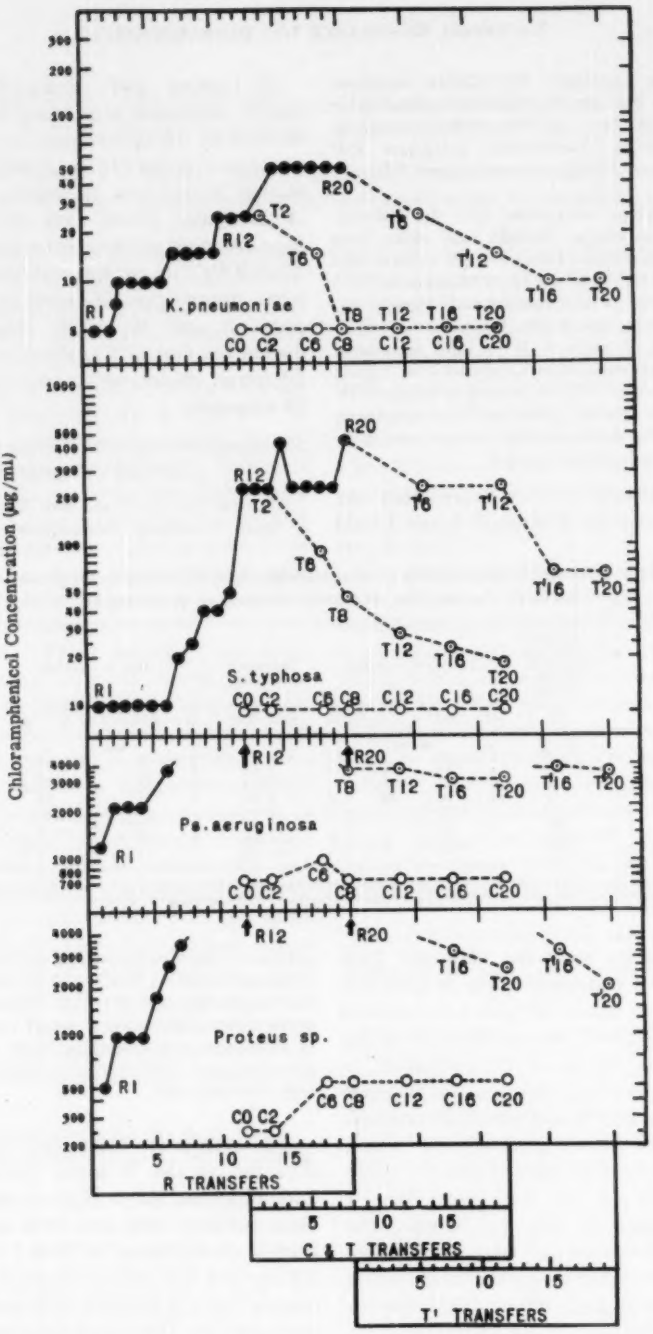

FIG. 1a.—Variations in concentration of chloramphenicol in broth required to inhibit visible growth of four bacterial cultures.

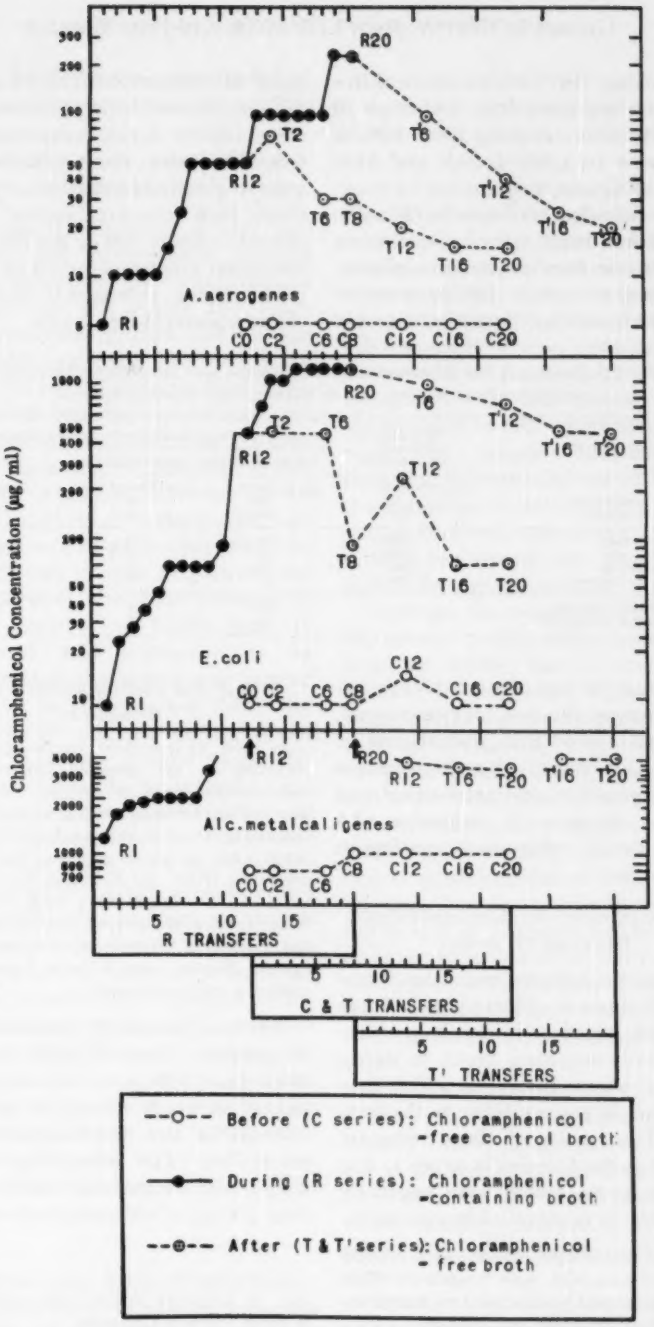

FIG. 1b.—Variations in concentration of chloramphenicol in broth required to inhibit visible growth of three bacterial cultures.

20 transfers in chloramphenicol-free broth starting from R12, and after 20 similar transfers starting from R20, is also shown in table 1 (6th and 11th colums of figures, respectively).

After repeated transfers in chloramphenicol-free broth, all of the cultures began to lose their acquired resistance. With one exception (*K. pneumoniae* after R12 transfers), the rate of loss was slower than the rate of increase of resistance.* Among the first four organisms, *Esch. coli* showed the greatest rate of increase (125 times) after 20 transfers in the presence of chloramphenicol and the least decrease of resistance (2.5 times) after 20 subsequent transfers in its absence.

Control transfers in chloramphenicol-free broth (C series)

The parent cultures were also transferred 20 times in chloramphenicol-free broth and tested at intervals in chloramphenicol-containing broth to determine the extent, if any, to which they might vary in susceptibility to the antibiotic. The results of these tests are depicted as the C series in figure 1. The first 4 cultures, which were originally susceptible to relatively low concentrations of chloramphenicol (5 or 10 μg per ml), showed little or no variation in susceptibility during subsequent transfers. The other three cultures, which were originally susceptible only to relatively high concentrations of chloramphenicol (250 or 750 μg per ml), showed somewhat greater variation in susceptibility during subsequent transfers in chloramphenicol-free broth.

TABLE 2.—*Decrease in free chloramphenicol concentration after incubating chloramphenicol-resistant cultures for three days in chloramphenicol-containing broth.*

Culture (R20)	Minimum inhibitory concentration	Free chloramphenicol concentration		
		Before incubation	After incubation	Decrease
	μg/ml broth	μg/ml broth	μg/ml filtered broth	Percent
K. pneumoniae	50	30	31	0 (+3)
A. aerogenes	250	30	30	0
S. typhosa	500	30	14	53
Esch. coli	1250	30	<2.5	>92
Proteus sp.	>4000	30	<1	>97
Ps. aeruginosa	>4000	30	12	60
Control (filtered)		30	27	10
Control (unfiltered)		30	36	0 (+20)

Loss of free chloramphenicol during incubation

In order to determine whether growth was occurring at the prepared chloramphenicol-concentration levels or whether free chloramphenicol was being lost during incubation, tubes containing 30 μg of chloramphenicol per ml of broth were inoculated with the final resistant organisms (R20) and incubated for three days at 37 C. The broths were then sterilized by Seitz filtration and assayed turbidimetrically by the method of Joslyn and Galbraith (1950) against *Shigella sonnei*.† Table 2 presents the results of this experiment.

The two cultures (*K. pneumoniae* and *A. aerogenes*) that were inhibited by not more than 250 μg of chloramphenicol per ml of broth caused no detectable decrease in free chloramphenicol concentrations. The other four cultures tested, which were inhibited by not less than 500 μg of chloramphenicol per ml

* Two of the resistant cultures, *Esch. coli* R20 and *A. aerogenes* R20, were lyophilized. When tested after several months, their resistance was found to have decreased only slightly.

† Turbidimetric assays were performed by Mrs. M. Galbraith in the research laboratories of Parke, Davis & Company.

of broth, decreased the concentration of free chloramphenicol by more than 50%.

Differences between susceptible and resistant cultures

While an investigation of the morphological and physiological changes that accompanied the acquisition of resistance was not within the scope of the present study, *Esch. coli* R20 was incidentally compared with the susceptible parent culture previous to lyophilization of the resistant subculture.*

No qualitative differences between the two cultures were observed in fermentation of glucose, lactose, saccharose, and citrate, or in the Voges-Proskauer, indole, and methyl red reactions. No differences in type of growth were noted between the two cultures in nutrient broth or on potato plug. In litmus milk, acid production by the parent culture was noted after several hours and became marked after four days, whereas acid production by the resistant culture became noticeable only after one week. No difference was noted in the Gram reaction, but the cells of the resistant culture were plumper and a few were much longer than those of the parent culture. After 6 and 26 hours, broth and agar cultures of the parent strain showed motility, but the corresponding resistant cultures did not. On nutrient agar, colonies of the parent culture formed rapidly and were uniformly smooth, round, entire, and translucent. Colonies of the resistant culture developed more slowly; half of them were hollow in the center, some appeared thin and rough and a few were smooth and opaque, with entire margins.

* Miss I. Ferguson of Parke, Davis & Company was kind enough to make the comparison and perform the lyophilization.

DISCUSSION

Bacteria have been shown to become more resistant to most antibiotics when they are maintained in medium containing the antibiotics. The present demonstration of this phenomenon for chloramphenicol is therefore not surprising. In vitro development of increased resistance, however, does not necessarily imply that this phenomenon will also occur in vivo. Penicillin-resistant strains, for example, have been developed in vitro but apparently have not emerged in vivo with sufficient frequency to interfere seriously with penicillin therapy. On the other hand, the emergence of streptomycin-resistant strains has been encountered not only in vitro but also in vivo when streptomycin therapy is prolonged.

Although the technique employed in the present studies differs from that of Demerec (1949), the chloramphenicol pattern, here revealed, appears to be more like his pattern for penicillin than that for streptomycin. In view of the similarity between the in vitro penicillin and chloramphenicol patterns, and of the relationship between in vitro and in vivo behavior with penicillin, prolonged administration of chloramphenicol may, likewise, prove to be free of the danger of emergent resistance. The demonstrated loss of in vitro acquired resistance upon subsequent propagation in the absence of chloramphenicol tends to strengthen the likelihood of this inference.

It is well known that the composition of the culture medium markedly affects growth rate and enzymatic activity. Since the results of the present study were obtained in a single medium, the extent to which variation in the medium might affect these results remains to be determined.

The chloramphenicol-containing broth cultures were incubated for 72 hours

because several preliminary cultures had been observed to reach maximum growth between 48 and 72 hours. Smith et al (1949b) have also reported that bacterial cultures containing quantities of the antibiotic sufficient to prevent growth could, when incubated for additional periods, overcome the bacteriostatic effects of the drug so as to resume normal growth. Such delayed growth may have become possible because of the demonstrated loss of free chloramphenicol during incubation.

There are at least two possible explanations for the mechanism of resistance. The organism may be able to develop a secondary metabolic pathway enabling it to circumvent those enzymatic reactions blocked by the antibiotic. This explanation may account for the results obtained with the more susceptible cultures which caused no detectable loss of free chloramphenicol. A second explanation for resistance may be found in the ability of an organism to increase the production of enzymes capable of degrading the antibiotic. The demonstrated loss of more than half of the free chloramphenicol in the presence of the more resistant bacteria may be due to degradation of the chloramphenicol molecule by enzymatic action of these bacteria. Smith et al (1949a and 1949b) have reported that susceptible cultures of *Esch. coli* and *Proteus*, resistant subcultures of which caused the greatest loss in these studies, produce enzymes capable of decreasing the characteristic antibacterial activity of chloramphenicol by reduction of the nitro group to a primary aromatic amine group and by hydrolysis of the amide linkage to liberate the corresponding basic aliphatic amine and dichloroacetic acid. Since Smith et al worked with relatively susceptible cultures, the ability of resistant subcultures to degrade chloramphenicol by these mechanisms has not been demonstrated and in any event is not a property that distinguishes resistant from susceptible cultures. Whether increased resistance is correlated with increased enzymatic activity and with possible additional chloramphenicol-inactivating mechanisms remains to be determined.

SUMMARY

When cultures representing 7 species of bacteria were transferred 20 times in chloramphenicol-containing broth, their resistance to the antibiotic was found to increase in a gradual fashion but not to the same extent. Upon subsequent transfer in chloramphenicol-free broth, their resistance decreased, but in 20 transfers did not return to the original levels.

More than half of the chloramphenicol added to cultures that grew in the presence of 500 or more μg of the antibiotic per ml was lost during incubation.

Changes accompanying increased resistance of one species are described.

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PRODUCTION OF STEAM VOLATILE ACIDS BY BACTERIA-FREE *ASCARIS LUMBRICOIDES*

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Almost fifty years ago Weinland[1] observed that aerobic and anearobic utilization of carbohydrate by *Ascaris lumbricoides* in vitro is associated with the production of large amounts of valeric acid. This author concluded that valeric acid is the major end product of fermentation of *Ascaris*[2]. While Weinland's findings have been confirmed,[3–6] his interpretations have been subjected to criticism. It has been pointed out[7–10] that the habitat of *Ascaris*, the host's intestinal tract, harbors a large number and a great variety of bacteria. Therefore, valeric acid could have been produced by the bacterial flora contaminating the helminth rather than by the nematode itself. Several bacteria are known which produce valeric acid, while the formation of large amounts of this acid by metazoa is very uncommon. Furthermore, the possibility cannot be excluded that metabolic products of *Ascaris* may be precursors for the bacterial synthesis of valeric acid. An unequivocal answer to this controversial question was sought through a study of the metabolism of *Ascaris* in the complete absence of bacteria.

Consequently, attempts have been made to sterilize *Ascaris* by the use of antibiotics. Several difficulties were anticipated with this procedure. Only small amounts of solutions containing antibiotics might reach the bacterial flora present in the intestinal tract of the nematode. Not all bacteria might be sensitive to any of the available antibiotics, and some of them might develop resistance. Furthermore, effective concentrations of some antibiotics might prove toxic to the helminths. However, under conditions described in this paper, bacteriologically sterile *Ascaris* can be obtained without apparent injury to the worm.

EXPERIMENTAL

Samples of *Ascaris lumbricoides* var. *suis* were removed from hog intestines, washed in sterile distilled water, ground aseptically and cultured in aerobic tryptose phosphate broth[11] containing 1% glucose and in anaerobic thioglycollate broth (Baltimore Biological Laboratories). From such mixtures of many worms three types of bacteria were isolated repeatedly: gram-positive cocci, gram-negative rods growing in large, coliform colonies (rod_1) and gram-negative rods growing in extremely small, elevated colonies (rod_2). The sen-

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sitivity of these organisms to various antibiotics was then determined in vitro[12] under aerobic and anaerobic conditions. Table 1 lists the respective sensitivity levels of the bacteria. At no time were any bacteria isolated which were not susceptible to at least one antibiotic.

On the basis of these results, mediums containing different antibiotics in concentrations considerably above their bactericidal level in vitro were tested for their ability to sterilize living *Ascaris*. The ascarids were removed from pig intestines with a minimum of contact with any of the surrounding packing equipment. Twenty to 30 worms were placed in 2 liters of a salt solution at 37 C for an initial washing. The salt solution, modified from that of Baldwin,[13] had the following composition: $1.4\times10^{-1}M$ NaCl, $2.7\times10^{-2}M$ KCl, $1.8\times10^{-2}M$ $CaCl_2$ and $4\times10^{-4}M$ $MgSO_4$; it was adjusted to pH 7.2 by the addition of dilute NaOH (2 to 3 ml of 0.125*N* NaOH are required for 2000 ml).

Parasites were then placed in a thermos jug containing 2 liters of the salt solution (37 C) and transported to the laboratory. Here the worms were transferred to a large beaker and washed for 30 minutes with tap water (37 C). Each individual worm was then removed and washed 4 times in 200 ml of the sterile salt solution. Following this final washing each *Ascaris* was allowed to remain for 48 hours at 37 C in 125 ml of a sterile salt solution to which antibiotics had been added. Subsequently, the parasites were transferred aseptically to 125 ml of the sterile (antibiotic-free) salt solution containing $1.25\times10^{-3}M$ phosphate buffer (pH 7.4) and incubated at 37 C for an additional 48 hours. After this period samples of the salt solution were added to the aerobic and anaerobic culture mediums. Each *Ascaris* was then removed from its solution and dissected with sterile instruments into segments of about 2 cm length. The segments were cut longitudinally, exposing the intestinal tract and the reproductive system, and placed in the aerobic and anaerobic culture mediums. Samples of these broths were then plated every 48 hours for 14 days on aerobic beef heart infusion agar and on anaerobic thioglycollate agar (1.5%) (Baltimore Biological Laboratories), respectively. If both the worm and its medium proved sterile consistently by the above technique, the remaining salt solution was analyzed for steam volatile acids.

TABLE 1.—*Minimal antibiotic concentrations effective against bacteria isolated from* Ascaris.

Bacteria	Penicillin units per ml	Streptomycin, μg per ml	Aureomycin, μg per ml	Chloramphenicol, μg per ml	Bacitracin units per ml
Gram-positive cocci	40				8
Gram-negative rods (rod_1)	40	15			20
Gram-negative rods (rod_2)	200	6	40	8	

Under these conditions 100 g of *Ascaris* produced 1.16 to 1.82 milliequivalents of steam volatile acids in 24 hours. Partition chromatography on buffered celite columns revealed that at least 38% of this material consisted of an acid or an isomeric mixture of acids containing 5 carbon atoms (valeric, isovaleric, methylethylacetic or trimethyl-

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acetic acid). Attempts to identify this fraction are now in progress. Over 10% of the steam volatile acids could be accounted for as fatty acids containing 6 carbons (caproic acid or its isomers). Acetic acid (14 to 25%) and smaller amounts (12% or less) of propionic and butyric acids could be detected regularly by partition chromatography. A somewhat similar distribution of steam volatile acids was found by Baldwin[14] in the body fluid of nonsterile *Ascaris*. If glucose was added to the sterile salt solution, the production of steam volatile acids by the helminths was increased to an extent of at least 100%, but the proportion of the acids containing 2 to 6 carbon atoms was about the same as in the absence of glucose. These results indicate that the production of valeric acid or of its isomers discovered by Weinland[1] is due to the metabolic activities of the nematode itself rather than to contaminating bacteria or to a symbiotic relationship between the latter and the helminth.

While no single antibiotic removed all bacteria from the parasite, effective sterilization of a significant number of worms could be obtained with combinations of antibiotics (table 2). In two series of experiments, sulfadiazine in low concentrations was added to the antibiotic mixture since it has been reported[15] that this compound acts synergistically with penicillin and with streptomycin. The level of antibiotics in all mixtures listed on table 2 is far below concentrations producing any signs of toxicity in the worm. Chloramphenicol (Chloromycetin) was the only antibiotic exhibiting any harmful effect on *Ascaris* as determined by worm motility and survival. With the exception of the latter antibiotic, bacteria-free worms survived for longer periods outside the host than did controls which had not been sterilized. For example, sterile *Ascaris* remained alive for more than two weeks in ox serum, while nonsterile worms died under similar conditions within four days. Thus, the use of antibiotics for the sterilization of *Ascaris* affords an opportunity for investigating the nutritional requirements as well as the metabolism of this parasite.

TABLE 2.—*Relative effectiveness of combinations of antibiotics in sterilizing* A. lumbricoides.

Penicillin units per ml	Streptomycin, μg per ml	Aureomycin, μg per ml	Bacitracin units per ml	Sulfadiazine, mg per ml	Number of worms tested	Percentage of sterile worms
1000					5	0
		200			20	5
			100		12	0
1000	500				10	20
1000		200			12	42
1000	500	200			15	40
1000	500		100		38	50
1000	500	200		1.2	40	55
1000	500		100	1.2	45	67

SUMMARY

1. A procedure is described by which living bacteria-free *Ascaris lumbricoides* can be obtained.

2. Bacteria-free ascarids produce considerable quantities of valeric acid and/or its isomers.

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ISOLATION OF THE VIRUS OF POLIOMYELITIS FROM THE STOOLS, OROPHARYNX AND NOSE OF CONTACTS

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The virus of poliomyelitis has been isolated from the stools of contacts on numerous occasions.[1,2] The majority of contacts excrete virus in the stools within a short period after exposure, particularly if they are young children[3] or, if adults, if the degree of exposure has been large,[4–8] e.g., parents of a clinically recognized case or others with prolonged exposure.

Virus has also been isolated, although much less frequently, from the oropharynx of contacts of clinical cases of poliomyelitis[6,9] and from the tonsils and adenoids of noncontacts.[9]

Virus isolation was reported from the nasal secretions of a child taken 4 months after the second paralytic attack.[10] However, the histopathology observed in the monkeys reported to have been infected following inoculation of the nasal secretions of this child would probably not be accepted today as conclusive evidence of infection with poliomyelitis virus.

More recently virus has been reported isolated from nasal swabs taken from a paralytic case[11] but contamination of the nose from the oropharynx could not be ruled out. Tests for virus in the nasal secretions of clinical cases are nearly always negative,[11,12] and isolation of virus from nasal secretions of contacts (either asymptomatic or subclinical) or from noncontacts has not been reported.

Comparative examination of stools and oropharynx of clinical cases for virus have been reported.[6,13,14] Similar comparative examinations have been done on contacts (both asymptomatic and with symptoms of subclinical poliomyelitis)[6,13,15–18] and on individuals with

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no known contact.[19] These studies have indicated that the virus is present in the oropharynx of clinical cases of poliomyelitis less frequently and, when present, persists for a shorter period of time than in the stools, although in one study in which stools were not tested, nearly 50% of the clinical cases tested during the first 3 days after the onset of symptoms had demonstrable virus in the oropharynx.[20] Likewise, virus is present in the oropharynx of subclinical cases less frequently than in the stools.

To our knowledge, comparative examinations of stools, oropharyngeal swabs and nasal swabs for the presence of poliomyelitis virus either in clinical or subclinical cases have not been reported.

In 1946 a case of paralytic poliomyelitis occurred in a day nursery school in Chicago which afforded the opportunity to examine a rather large group of child contacts for the presence of virus. This report is concerned with the results of that study.

MATERIALS AND METHODS

Following the appearance of the paralytic case careful clinical observations were made daily on the other children in the nursery school, and, whenever possible, specimens were collected for virus study.

The collection, storage and preparation for inoculation into monkeys of the stools and oropharyngeal swabs were done exactly as reported previously.[2,9] Briefly, this consisted of storage of daily stools and oropharyngeal swabs in 50% glycerine-0.9% sodium chloride solution in solid carbon dioxide refrigerators until used. Several of each kind of specimen from each child were pooled, cleared by centrifugalization and shaken with absolute diethyl ether on successive days until viable bacteria in the specimens were few or absent.

Nasal swabs were collected by turning a dry cotton-tipped applicator in the nose bilaterally and placing the cotton-tipped end in a vial containing 50% glycerine-0.9% sodium chloride solution and storing as described above until used. Nasal swabs were prepared for monkey inoculation exactly as the oropharyngeal swabs.[9]

Specimens were selected for testing from those collected from the onset of symptoms until a few days after onset among those children developing recognizable symptoms. Specimens from the 10 children who did not develop recognizable symptoms were selected for testing from those dates on which the other children were showing recognizable symptoms, and in no case extended over an interval greater than 18 days. The collection intervals listed in table 1 are the inclusive dates of specimens of all types tested and in most cases do not represent pools of specimens from each day in the time interval shown.

Two monkeys, one rhesus (*Macaca mulatta*) and one cynomolgus (*Macaca cynomolgus*), were inoculated with the pools of each type of specimen from each child. Each animal was inoculated intracerebrally and intraperitoneally with the ether-treated material and intranasally with "raw" or untreated material. The technique of inoculation, observation of the animals, autopsy and histopathologic examination of central nervous system sections* were the same as previously described.[2,9]

Fever, progressive paralysis and typical microscopic lesions in the central nervous system were observed in all inoculated monkeys where a positive result is recorded.

Nine children residing in the vicinity of the nursery school but having no direct contact with the children in the school or with any other known source of poliomyelitis virus were also studied. Clinical observations were made on these children daily and stools, oropharyngeal swabs and nasal swabs were collected for virus study. These specimens were tested for the presence of virus in the same manner as described above for those from the contact children. These children are designated as noncontacts in table 1.

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* We are indebted to Miss Erma Salter for the preparation of the histologic sections.

RESULTS

Of the 40 children in the nursery school at the time of the appearance of the paralytic case, 29 were available for study. The others moved from the neighborhood, went on vacations or for other reasons were not present at the time of the investigation. One of the 29 children was the original paralytic case in the school, and 2 others subsequently developed symptoms of meningitic poliomyelitis (stiff neck among other symptoms). Complete testing of stools, oropharyngeal swabs and nasal swabs was not done on these children who developed clinical cases of poliomyelitis (paralytic and meningitic) because our primary purpose was to test the virus distribution in contacts who did not have the paralytic or meningitic disease. However, stools and oropharyngeal swabs from one of the meningitic cases were tested for virus.

The clinical observations and the results of the monkey inoculations are recorded in table 1.

TABLE 1.—*Recovery of virus from child contacts and noncontacts of a paralytic case of poliomyelitis occurring in a day nursery school.*

Color	Sex	Age	Onset of symptoms	Symptoms*	Spinal fluid findings	Collection dates of specimens for virus tests	Virus isolations		
							Stool	Oro-pharynx	Nose
		Contacts							
W†	M	2½	7/17/46	P, F, W, LA, H, SN, SB, ST, V, C, DR, I					
W‡	M	4½	7/25/46	F, SN, ST, V, C	+				
W‡	M	5	8/ 5/46	F, H, SN, C, P	+	7/30–8/ 1/46	+	+	
W	F	2½	8/ 5/46	F	0	7/27–8/ 7/46	+	0	0
W	F	3	7/22/46	F, LA, D, DR	0	7/26–8/ 5/46	+	+	0
W	M	5½	7/22/46	F, LA, H, D, RN, DR	0	7/26–8/ 8/46	+	+	+
W	M	4	None	None	0	7/29–7/31/46	0	+	0
W	F	2½	7/22/46	F, H, ST		7/26–8/ 5/46	+	+	0
W	M	4	7/18/46	RN, CG	0	7/26–8/ 6/46	+	0	0
W	F	3	8/15/46	F, RN, DR		8/14–8/21/46	+	0	0
W	M	5	None	None		8/13–8/21/46	0	0	0
W	M	6	7/26/46	F, W, H, DR		7/26–8/ 9/46	0	0	0
W	M	3	7/29/46	F, W, H, ST, V, C, D, RN, CG	0	7/27–8/ 8/46	+	0	0
W	M	4	7/21/46	F, H, C, RN		7/26–7/31/46	+	0	0
W	M	2½	7/27/46	F		7/27–8/ 8/46	+	+	0
W	M	4	7/25/46	RN		7/26–8/13/46	+	0	0
W	F	4½	7/23/46	V, C		7/29–7/31/46	+	0	0
W	F	3½	None	None		7/26–7/31/46	0	0	0
W	M	3	8/ 5/46	F	0	7/29–8/ 6/46	+	0	0
W	F	5½	None	None	+	7/29–8/ 6/46	+	0	0
W	F	5	7/25/46	F, ST, V, DR	+	7/26–8/ 6/46	0	0	0
W	M	3½	7/28/46	F, C, DR	0	7/26–8/ 8/46	+	0	0
W	M	3½	8/ 2/46	F		8/ 3–8/ 8/46	0	0	0
W	M	2	None	None		7/27–8/ 8/46	+	0	0
W	F	2	7/20/46	F, H, V, C, RN, I, FR	+	7/26–8/ 5/46	+	0	0
W	M	3½	7/31/46	C, VC		7/31–8/12/46	+	0	0
W	M	3½	None	None		7/27–8/ 7/46	+	0	0
W	M	4½	None	None	0	7/27–8/ 7/46	+	0	0
W	F	4	7/29/46	F, ST, CG		7/29–8/ 1/46	+	0	0
		Noncontacts							
W	M	4	None	None		8/2 –8/ 8/46	+	0	0
W	M	10	8/ 1/46	C, DR		8/ 7–8/ 8/46	0	0	0
W	F	1½	7/31/46	RN		8/ 1–8/ 8/46	0	0	0
W	M	4	8/ 9/46	LA		8/ 6–8/ 7/46	0	0	0
W	F	3½	8/ 7/46	RN, CG		8/ 2–8/ 8/46	0	0	0
W	M	8	None	None		8/ 2–8/ 8/46	0	0	0
W	F	2	None	None		8/ 1–8/ 8/46	0	0	0
W	M	9½	None	None		8/ 2–8/ 8/46	0	0	0
W	F	3	8/ 6/46	ST		8/ 1–8/ 8/46	0	0	0

* F—fever, P—paralysis, W—weakness, LA—leg ache, H—headache, SN—stiff neck, SB—stiff back, ST—sore throat, V—vomiting, C—constipation, D—diarrhea, RN—running nose, CG—cough, DR—drowsiness, I—irritability, FR—frequency of urination, VC—voice changes.

† This child was the paralytic case which called our attention to the nursery school.

‡ These two children were the recognized clinical cases among the contacts of the first child listed.

Of the 26 contact children studied who did not develop paralytic or meningitic poliomyelitis, 16 developed one or more symptoms compatible with subclinical poliomyelitis.[21] In addition,

21. Casey, A. E., Fishbein, W. I., Gordon, F. B., Schabel, F. M., Jr. and Bundesen, H. N. 1948, J.A.M.A. **138:** 865–868.

2 of these 16 with and 1 without symptoms of subclinical poliomyelitis had spinal fluid findings compatible with a central nervous system infection.[22] Thus, 17 of the contact children were suspected of having had subclinical poliomyelitis on the basis of clinical observations and spinal fluid findings.

Virus was isolated from the stools, oropharyngeal swabs, and/or nasal swabs of 22 of the 27 contact children studied.

Of the 28 contacts in the school, 2 had clinically recognized poliomyelitis (meningitic) and 21 of the 26 other contacts, who were either asymptomatic or had symptoms compatible with subclinical poliomyelitis, harbored virus in their stools, oropharynx and/or nose.

Among the 5 contacts tested from whom virus was not isolated, one child had symptoms compatible with subclinical poliomyelitis and spinal fluid findings indicative of a central nervous system infection, and another had symptoms of subclinical poliomyelitis but spinal fluid findings were negative. On the basis of known exposure and observed symptoms, it is probable that these 2 children also had subclinical poliomyelitis. The remaining 3 children from whom virus was not isolated had either minor symptoms or were asymptomatic and were considered to have escaped infection following this exposure to the virus. It is possible that they may have had a subclinical infection sometime earlier in life.[3]

On the basis of clinically recognized infections and virus isolations, 23 of the 28 (82%) child contacts had poliomyelitis, and probably 2 more had the subclinical disease, indicating an incidence of infection with poliomyelitis of 25 of the 28 (89%) of the child contacts in the nursery school.

Virus was isolated from 2 contacts in whom no symptoms compatible with subclinical poliomyelitis[21] were observed and on whom spinal fluid studies were not done.

Virus was isolated from the stools of 21 contacts. One of these had meningitic poliomyelitis, 14 had symptoms compatible with subclinical poliomyelitis, 2 had minor symptoms and 4 had no observed symptoms.

Virus was isolated from the oropharyngeal swabs of 6 contacts. One of these had meningitic poliomyelitis, 3 had symptoms compatible with subclinical poliomyelitis, 1 had minor symptoms and 1 had no observed symptoms. This latter child did not have detectable virus in the stools taken at the same time as the oropharyngeal swabs.

Virus was isolated from the nasal swabs of one of the contacts. This child also was shown to be harboring virus in both the stools and oropharynx.

One of the 9 (11%) noncontact children was shown to be harboring virus in the stools.

DISCUSSION

Data presented here indicate that under favorable conditions, e.g., good contact and a susceptible population, a high percentage of those exposed to poliomyelitis virus become infected. The nursery school study reported here is similar to the reports of extensive infection among contacts in a boys' camp,[12] a children's home,[7] and among familial contacts,[2,4,5,15,16] to cite only a few of the most striking. Individuals with the subclinical disease have been shown to be excreting virus in the stools in a considerable percentage of cases for several weeks after the onset of the infection.[23]

22. Andelman, M. B., Fishbein, W. I., Casey, A. E. and Bundesen, H. N. 1946, South Med. J. **39**: 706–718.

23. Schabel, F. M., Jr., Smith, H. T., Fishbein, W. I. and Casey, A. E. 1950, J. Infect. Dis. **86**: 214–218.

The isolation of virus from the stools of asymptomatic contacts has been reported previously.[13,15–17]

There is considerable controversy regarding the significance of presence of virus in the oropharynx. It is present there in a considerable number of clinical cases, particularly during a short period after onset of symptoms.[16,20] The apparent fact that the presence of virus in the oropharynx approximates the epidemiologically observed infectious period of cases has been suggested as having possible significance in the epidemiology of the disease.[9]

In addition to the time interval in relation to onset in which virus has been observed in the oropharynx of poliomyelitis cases, Casey et al[18] have concluded that its presence there is also related to observed symptoms, virus being present more frequently in those individuals with well marked symptoms, whether the case is clinical (paralytic or meningitic) or subclinical.

Wenner and Tanner[16] also have concluded "it would appear that time is the essence of importance in sampling (throats)," but on the other hand they concluded "that clinical gravity of illness is of little significance in regard to the presence of poliomyelitis virus in the oropharynx."

Aside from the controversial aspects of observations on oropharyngeal poliomyelitis virus, the fact that the virus has been shown to be present in the oropharynx of subclinical cases and asymptomatic individuals by virus studies as reported here and by others,[15–17,19,24] and the fact that virus was probably present in the oropharynx in the numerous cases of bulbar paralysis reported following tonsillectomy and adenoidectomy,[1] indicate the great importance of virus being present in this site, particularly in subclinical cases.

We are inclined to attach little significance to the detection of virus in the nasal swabs of the one child reported here. Virus has been isolated only very rarely from the nose of clinical cases, and histopathologic examination of olfactory bulbs of fatal cases and epidemiologic observations on chemical blockade of olfactory pathways have indicated that the nose plays an unimportant role in naturally occurring poliomyelitis infections of man. The fact that the child reported here to have had virus in its nasal swabs also had detectable stool and oropharyngeal virus in addition to well defined symptoms of subclinical poliomyelitis indicates that the infection was widespread, and, while the virus may have been present in the nose in the natural course of the infection, we feel that the possibility of contamination of the nose from the oropharynx cannot be dismissed lightly.

The incidence of infection of 11% among the noncontacts studied is in agreement with other reports[2,3] and indicates that there was no widespread infection in the neighborhood caused by some environmental factor common to all. The data presented do not prove conclusively that the first or only contact with the virus of poliomyelitis by the children in the school was the paralytic case listed in table 1. They do indicate, however, that the source of virus was probably in the school and not widespread in the neighborhood, and they further indicate that the contact with the virus by the children in the school probably occurred during a short period of time.

SUMMARY

Among the 28 contacts of a paralytic case of poliomyelitis in a nursery school

24. Howe, H. A. and Bodian, D. 1947, Am. J. Hyg. **45**: 219–222.

in Chicago in 1946, virus was isolated from the stools, oropharyngeal swabs and/or nasal swabs of 22, and 1 child who was not tested for the presence of virus developed a clinically recognized case of meningitic poliomyelitis. Two of the children from whom virus was not isolated had symptoms compatible with subclinical poliomyelitis. Thus, 25 of the 28 (89%) contacts of preschool age developed poliomyelitis. Three of the children had neither symptoms nor demonstrable virus in the specimens tested and were considered to have escaped infection from this particular exposure.

Virus was isolated from the stools of 21 contacts (78%). One of these had meningitic poliomyelitis, 14 had symptoms compatible with subclinical poliomyelitis, 2 had minor symptoms and 4 had no observed symptoms.

Virus was isolated from the oropharyngeal swabs of 6 of the contacts (22%); 1 had meningitic poliomyelitis, 3 had symptoms comparable with subclinical poliomyelitis, 1 had minor symptoms and 1 had no observed symptoms. The latter child did not have detectable virus in the stools taken at the same time as the oropharyngeal swabs.

Virus was isolated from the nasal swabs of 1 contact (4%), but the stools and oropharyngeal swabs of this child taken at the same time as the nasal swabs likewise contained virus, and nasal contamination from some other site could not be ruled out.

One of 9 (11%) of noncontact children tested had demonstrable virus in the stools.

THE EFFECT OF X-IRRADIATION ON EXPERIMENTAL ENTERIC CHOLERA IN THE GUINEA PIG

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In the precise sense of an enteric infection the causative microorganism proliferates within the lumen of the bowel, invading only the superficial layers of the intestinal mucosa, and the infection remains essentially outside the tissues of the body. Of those infections which give rise to disease, Asiatic cholera in man is probably the most clear-cut example. The presence of the normal bacterial flora of the intestine may perhaps be regarded as an infection, or group of infections, which do not ordinarily result in overt symptoms of disease.

It was originally shown by Koch in connection with studies on the etiology of human cholera that intragastric inoculation of the guinea pig with massive doses of *Vibrio cholerae* following parenteral administration of tincture of opium resulted in death in 24 to 48 hours. In retrospect it seems probable that the cause of death was toxemia. Subsequent investigation by Burrows, Elliott and Havens (1947) showed, however, that a true, but symptomless and nonlethal, infection could be produced in which the vibrios multiplied within the bowel and were only occasionally found in the tissues and then only in the spleen. The experimental enteric infection is thus a useful model, particularly since neither the cholera vibrio nor related forms which may be confused with it are naturally occurring parasites of the guinea pig. Crecelius and Rettger (1943) have, in fact, shown that the intestinal flora of the guinea pig is not predominately coliform in character but consists largely of gram-positive bacilli of the genus *Lactobacillus*, together with smaller numbers of an obligate anaerobic sarcina; in our own studies three colonial types of alkali-tolerant micrococci have been encountered consistently and in large numbers on Vedder and van Dam medium cultures. Furthermore, the guinea pig is highly susceptible to parenteral inoculation with the cholera vibrio, the basis of much of the work on "intraperitoneal cholera" of Pfeiffer and others, and spread from the bowel often results in a fatal generalized infection. Bacteriologically the enteric infection is characterized by the excretion of enormous numbers of vibrios in the feces in the first few days after infection; these then decrease relatively rapidly and disappear 10 to 20 days after inoculation. The intensity of the infection is most readily assayed as the percentage of vibrios of the total fecal flora cultivable on the selective alkaline medium used for their isolation.

In spite of a reasonably effective physical separation of the focus of infection from tissue antibody, the specific immune response of man to prophylactic cholera vaccine or to naturally occurring infection appears to be an effective one in that, on the one hand, an appreciable degree of protection is conferred by immunization (Adiseshan, Pandit and Venkatraman, 1947), and, on the other, the vibrios disappear rapidly from the feces during

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convalescence. It has been shown by Burrows, Elliott and Havens (1947) that an immunity to massive doses of vibrios, evidenced by a sharp reduction in numbers excreted in the feces and a shortening in the duration of the infection in animals actively immunized with vaccine or by a prior infection, is associated with the presence of antibody in the feces. It has also been found (Burrows and Ware, 1949; Burrows, Ware and Deupree, 1950) that an effective prophylactic immunity in the guinea pig, measured as an increased minimal infective dose, is similarly associated with the coproantibody response to active immunization.

Barksdale and Gohda (1950a) have reported that man forms coproantibody to homologous coliform flora of the intestine, and we have observed a similar immune response (unpublished observations). Barksdale and Gohda (1950b) have also found that food-borne *Salmonella paratyphi B* infection of immunized persons resulted in a carrier state in which these bacteria temporarily assumed the status of normal fecal flora in the individuals affected, and have observed too that antibody to the O, H and Vi antigens of the typhoid bacillus occurs in the feces of chronic typhoid carriers. We too have found evidence of coproantibody response to the typhoid bacillus in the typhoid carrier (unpublished observations).

Although as yet fragmentary, the foregoing observations suggest that coproantibody may be functional in acquired immunity to enteric infection with pathogenic bacteria, and as well possibly constitutes a significant facet of natural immunity in the maintenance of an equilibrium between host and parasitic intestinal flora.

It has been reported (Burrows, Elliott and Havens, 1947) that if actively immunized guinea pigs were infected after coproantibody had disappeared but appreciable serum antibody titer persisted, they behaved as though nonimmune to the experimental infection. Experiments on the effect of X-irradiation on the coproantibody response carried on concurrently and described elsewhere (Burrows, Deupree and Moore, 1950), indicated that postimmunization irradiation with doses of 100 to 150r given while antibody titer was rising and near its peak, partially or completely inhibited coproantibody though serum antibody titers were not significantly affected. It was, therefore, of interest to determine whether or not actively immunized animals deprived selectively of coproantibody by postimmunization X-irradiation would also react as nonimmunes to infection. An essential preliminary, however, is a study of the infection in nonimmune irradiated animals. This consideration prompted in part the investigation reported here; additional studies on irradiated immunized animals will be reported in a subsequent publication.

In addition, experimental enteric cholera in the guinea pig provides, as pointed out above, a useful model. Since the infection is symptomless and nonfatal, it may be regarded as related in some degree to the symptomless infection of a host with its normal intestinal flora, except that the cholera vibrio is rapidly eliminated while the normal bacterial flora of the bowel presumably is not. The last is not a complete difference, however, since, for example, Wallick and Stuart (1943) have shown that some individuals at least carry a succession of immunologic types of coliform bacilli.

METHODS

The strain of *V. cholerae* used for infection and as an agglutinating antigen was NIH no. 41, the Ogawa vaccine strain. It had been received from the National Institute of Health in 1942, main-

TABLE 1.—*Autopsy cultures of normal animals.*

Day	Animal		Cultures				
			Peritoneal fluid	Spleen	Liver	Kidney	Heart's blood
1	xa	1	—	—	—	—	—
	xa	23	—	—	—	—	—
3	xa	4	—	—	—	—	—
	xa	27	—	—	—	—	—
5	xa	7	—	—	—	—	—
	xa	30	—	—	—	—	—
7	xa	10	—	—	—	—	—
	xa	33	—	*Staph. aureus*	—	—	*Staph. aureus*
	xa	w7*	—	β strep	—	—	β strep
9	xa	13	—	—	β strep	—	—
	xa	36	—	*Staph. aureus*	—	—	—
11	xa	16	—	—	—	—	—
	xa	39	—	—	—	—	*Staph. aureus*
12	xa	w8*	—	β strep	—	—	β strep
13	xa	42	—	—	—	—	—
15	xa	45	—	—	—	*Staph. aureus*	*Staph. aureus*

* Moribund when killed.

TABLE 2.—*Autopsy cultures of infected animals.*

Day	Animal		Cultures						
			Duodenum	Ileum	Peritoneal fluid	Spleen	Liver	Kidney	Heart's blood
1	xa	2	Cholera	Cholera	—	—	—	—	—
	xa	3	Cholera	Cholera	—	—	—	—	—
	xa	24	—	—	—	—	—	—	—
	xa	25	—	—	—	—	—	—	—
3	xa	5	Cholera	—	—	—	—	—	—
	xa	6	—	—	—	—	—	—	—
	xa	26*	Cholera	Cholera	—	—	—	—	—
	xa	28	Cholera	Cholera	—	—	—	—	—
	xa	29	Cholera	Cholera	—	—	—	—	β strep
5	xa	8	Cholera	Cholera	—	—	β strep	—	β strep
	xa	9	Cholera	Cholera	—	—	—	—	β strep
	xa	31	Cholera	Cholera	—	—	—	—	—
	xa	32	Cholera	Cholera	—	—	—	—	—
7	xa	11	Cholera	Cholera	—	—	—	—	—
	xa	12*	Cholera	Cholera	—	Cholera	Cholera	—	Cholera
	xa	34	Cholera	Cholera	—	—	—	*Staph. aureus*	—
	xa	35	Cholera	Cholera	—	—	—	—	—
	xa	w1*	—	—	*Staph. albus*	—	—	*Staph. albus*	—
9	xa	14	—	—	—	—	—	—	—
	xa	15	Cholera	Cholera	—	—	Cholera	—	—
	xa	37	Cholera	Cholera	—	—	—	*Staph. aureus*	*Staph. aureus*
	xa	38	Cholera	Cholera	—	—	—	Cholera	Cholera
	xa	w2*	Cholera	Cholera	β strep	β strep	β strep	β strep	β strep
11	xa	17	Cholera	Cholera	—	—	—	*Staph. aureus*	
	xa	18*	Cholera	Cholera	—	Cholera	Cholera	Cholera	Cholera
	xa	40	Cholera	Cholera	—	—	—	Cholera	—
	xa	41	Cholera	Cholera	—	—	—	*Staph. aureus*	*Staph. aureus*
	xa	w3*	Cholera	Cholera	—	—	—	*Staph. aureus*	*Staph. aureus*
	xa	w4*	Cholera	Cholera	—	β strep	β strep	—	β strep
13	xa	19	Cholera	Cholera	—	—	—	—	—
	xa	20	Cholera	Cholera	—	—	—	—	—
	xa	43*	Cholera	Cholera	—	—	Cholera	Cholera	Cholera
	xa	44	Cholera	Cholera	—	—	—	Cholera	—
	xa	w5*	Cholera	Cholera	β strep	β strep	β strep	β strep	β strep
15	xa	21	Cholera	Cholera	—	β strep	β strep	β strep	β strep
	xa	22	Cholera	Cholera	—	—	—	—	Cholera
	xa	46	Cholera	Cholera	—	—	—	—	—
	xa	47*	Cholera	Cholera	—	Cholera	Cholera	Cholera	Cholera
17	xa	48	Cholera	Cholera	—	Cholera	—	Cholera	—
	xa	49	Cholera	Cholera	—	—	Cholera	Cholera	—
	xa	w6*	Cholera	Cholera	β strep	β strep	β strep	—	β strep
19	xa	60	Cholera	Cholera	—	Cholera	Cholera	Cholera	—
	xa	51	Cholera	Cholera	—	—	—	—	Cholera

* Moribund when killed.

tained in lyophilized form, and carried on blood agar during the course of these experiments.

Guinea pigs were young animals of inbred stock, weighing 300 to 400 g. They were received a week or more before infection and kept on the stock diet of commercially prepared pellets, cabbage and carrots. A short time prior to infection the animals were given 2 mg morphine sulfate in saline solution by the intraperitoneal route. The inoculum was a suspension of 18-hour growth of vibrios on veal infusion agar, pH 8, suspended in isotonic phosphate buffer of pH 8 of a density such that the 2 ml of inoculum contained the equivalent of 20 mg dry weight of bacteria. The bacterial suspension was given intragastrically, using a soft rubber catheter attached to a syringe as a stomach tube.

The animals were given whole body irradiation in doses ranging from 100 to 200 r under the following conditions: 200 KV, 15 milliamperes, 0.5 mm copper and 3 mm bakelite filter, target distance 28.5 inches, roentgen output 17 r per minute.

In several series of experiments irradiated animals, both infected and not infected, were sacrificed on alternate days. Those sacrificed were selected in that animals obviously ill or moribund were chosen. After killing by a sharp blow on the back of the neck, the animals were autopsied and cultures taken of the peritoneal fluid, spleen, liver, kidney, heart's blood, and intestinal contents high and low in the small intestine. In the case of the organs a freshly cut surface was used to inoculate culture mediums. All specimens were cultured on blood agar and on Vedder and van Dam's glycine-buffered hemoglobin medium. Colonies suspected of being *V. cholerae* were examined in gram-stained smears, and tested against homologous O antiserum by slide agglutination. Aside from cultures of intestinal contents, all showed only staphylococci or streptococci or cholera vibrios. Staphylococci were identified only by gram-stain morphology and pigment production; none of the *albus* strains and all of the *aureus* strains were β-hemolytic. The streptococci were all β-hemolytic and were identified for present purposes on the basis of this and microscopic morphology in gram-stained smears. Neither staphylococci nor streptococci grew on the Vedder and van Dam medium, but cholera vibrios grew on both this and on blood agar.

Twenty-four-hour fecal specimens were collected on alternate days in a modified metabolism cage in 30 ml of isotonic phosphate buffer, pH 8. Urine and feces were effectively separated in an inclined bifurcated funnel in which a brass screen was set at an angle. Urine specimens were discarded. During collection periods the animals were fed only carrots ad libitum. The fecal pellets were shaken to a uniform suspension in a shaking machine, aliquots removed for quantitative dilution and plate culture on Vedder and van Dam medium, and formalin added to the remainder to 0.5%. The formalinized suspension was clarified by high speed centrifugation and the supernate decanted and used for antibody titration. After 48 hours incubation the plates were counted under a dissecting microscope, and the proportion of cholera vibrio colonies calculated as a percentage of the total count. Fecal specimens were collected from groups of 6 animals, the original group being supplemented as animals died from an infected irradiated pool. In groups of 6 variability in the bacterial counts is reduced to reasonable proportions.

The O agglutinin in the fecal extracts was titrated against a refluxed O antigen. The O antibody was selectively titrated since it is protective (Burrows, Mather, Elliott and Havens, 1947). Details of the method of antibody titration are described elsewhere (Burrows, Mather, McGann and Wagner, 1946).

RESULTS

Two groups of 70 animals each given 200r X-radiation and infected the following day provided pools from which representatives were taken on alternate days for autopsy and culture. Another group of 40 animals that were irradiated but not infected served as controls. Of the total number, many died during the night and were discarded. All the pools were exhausted during the experiments, the first two at 17 and 19 days, and the control group at 15 days. The results of culture are given in the accompanying tables, table 1 showing the irradiated, noninfected control group, and table 2 a composite of the two groups of infected, irradiated animals. While the total number of animals examined is too small for valid statistical examination, the following summations may be made: Of the 16 control animals autopsied, 7 were found to be infected, and of these 4 showed *Staphylococcus aureus* infection and the remaining 3 β-hemolytic streptococcus infection. Of the 7, 5 gave positive heart's blood cul-

tures indicating bacteremia. Of the 43 infected animals autopsied, 27 were infected, 8 with β-hemolytic streptococcus, 1 with *Staphylococcus albus* and 5 with *Staph. aureus*, and 13 with the cholera vibrio.

The high proportion of cholera infections, almost as large as the staphylococcus and streptococcus infections combined, is perhaps to be regarded as a reflection of its pathogenicity for the guinea pig once it has gained entrance to the tissues just as the predominance of *Staph. aureus* over *Staph. albus* is no doubt a consequence of the well known greater virulence of the β-hemolytic *aureus* strains. Failure to find disseminated infection with gram-negative bacilli of the enteric group is probably due to the absence of these forms in the normal intestinal flora of the guinea pig. Comparison of these data with the results of autopsy culture of infected but nonirradiated animals (Burrows, Elliott and Havens, 1947) clearly shows the effect of irradiation in enhancing spread of the experimental enteric infection from the lumen of the bowel into the tissues, beginning about a week after irradiation. An additional, and perhaps

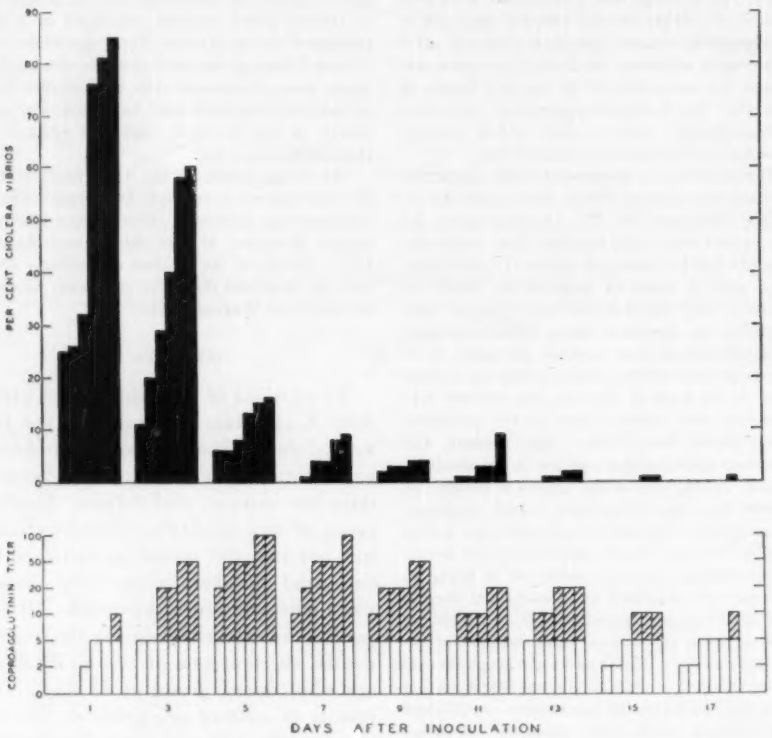

FIG. 1.—The course of enteric cholera in the guinea pig produced by a dose of 20 mg given intragastrically and assayed as percentage cholera vibrios of the total cultivable fecal flora and coproagglutinin titer.

significant, point is the failure to culture the cholera vibrio from the intestinal contents in 3 of 9 infected animals on the first and third days following infection when, in the normal animal, the numbers excreted are at a maximum.

The foregoing approach to the effects of irradiation on enteric cholera infection was supplemented by a series of experiments in which the progress of the experimental infection in animals receiving varying doses of X-radiation was followed by bacterial counts of excreted vibrios and titration of coproantibody. The course of infection produced by a dose of 20 mg of cholera vibrios in the normal animal is illustrated in figure 1. The pattern of vibrio excretion is characteristic, with large numbers excreted in the early days of the infection, followed by progressive decline and disappearance of the bacteria, only one animal remaining infected at the 7th day. Coproantibody response to the infection is apparent by the 3rd day, reaching a peak on the 5th day coincident with a sharp decline in numbers of vibrios excreted.

Results representative of those obtained in a series of experiments with animals irradiated with 200r the day prior to infection are shown in figure 2. It is clearly apparent that both the progress of the infection and the coproantibody response differ from those observed in the normal animal. Regarding the former, the infection is sharply modified in the first three days in that the number of vibrios excreted in much less than in the normal animal. It is to be noted also that of six animals infected, only three excreted detectable numbers of cholera vibrios on the first day, and only two on the third day, an observation consistent with the failure to culture vibrios from the intestinal contents of some of the infected animals at autopsy in the first three days of infection as described above. Subsequently the infection increases in severity as evidenced by a rise in the numbers of vibrios excreted, coinciding

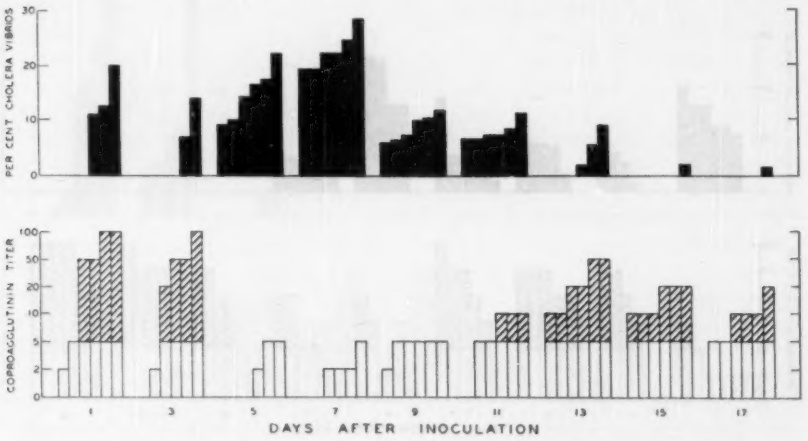

FIG. 2.—The effect of 200r X-irradiation given the day before infection on enteric cholera and coproantibody response to the infection. The inverse relation between the intensity of the infection and antibody titer is evident.

in time with its generalization as indicated by the results of autopsy culture and with the appearance of tissue invasion. There is also a sharp contrast between the normal and irradiated animals in the behavior of coproantibody in that in the irradiated animals it reaches peak titer on the first day after infection, remains high though declining somewhat on the third day, and disappears on the fifth day coincident with flare-up and generalization of the enteric infection. The secondary coproantibody response apparent on the eleventh day associated with subsidence and elimination of infection in the survivors no doubt represents a normal immune response to continued presence of the antigen as the animals begin to recover from the effects of irradiation. In a comparison of this approach to the infection and that of periodic sacrifice and autopsy culture, it must be emphasized that selection plays an important part. In these experiments survivors are necessarily selected, while autopsy of ill and moribund animals as well as those showing no symptoms of irradiation sickness tends to select those which do not survive.

The dosage of X-radiation required to so modify the enteric infection was examined in a series of experiments in which the animals received 150r and 100r the day prior to infection. Typical results of irradiation with 150r are shown in figure 3, and of irradiation with 100r in figure 4. The same kind of effect of irradiation as that observed with 200r is apparent in figure 3 but is less marked. Thus the numbers of vibrios excreted early is reduced over the normal, but all animals in this series were infected and the depressing effect persists through the 7th day instead of only through the 3rd with a flare-up on one day only, the 7th, and complete elimination of the infection by the 13th day as contrasted with the 17th in both normal and animals irradiated with 200r. Initial stimulation of the coproantibody response is not marked, but depression at the 9th day coincident with increased intensity of the infection

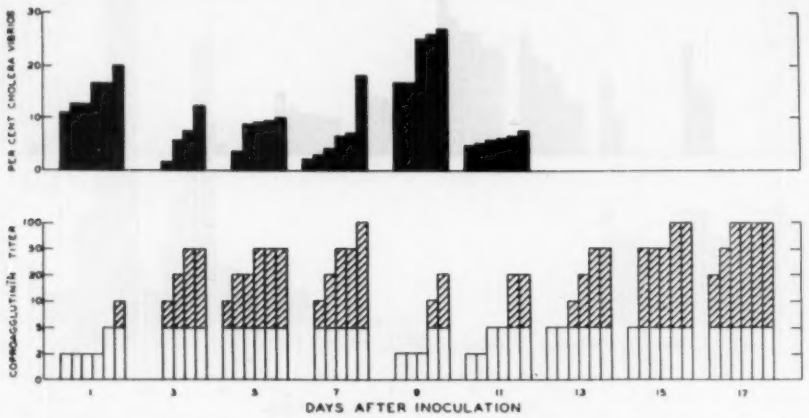

FIG. 3.—The effect of 150r X-irradiation on subsequently induced enteric cholera and coproagglutinin response to the infection.

is only partial, and the secondary immune response is stronger than that in animals receiving 200r. As shown in figure 4, a dose of 100r still results in a reduction in the severity of the infection during the first three days over that in the normal, but shortens its duration with only two animals infected on the 5th day and none thereafter. Copro-

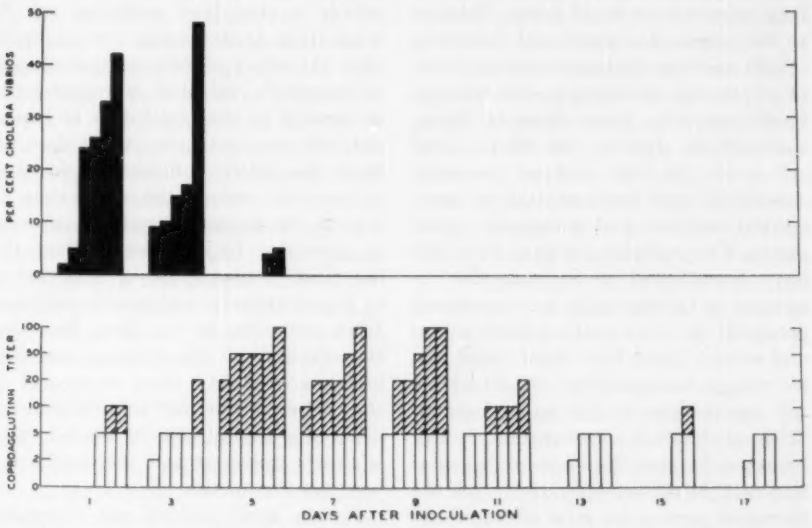

FIG. 4.—The effect of 100r X-irradiation on subsequently induced enteric cholera. The therapeutic effect of irradiation on the course of the infection is apparent.

antibody response falls off more rapidly than in the normal, possibly a consequence of a lessened persistence of the infection and therefore antigenic stimulus. In general the effect of irradiation with 100r appears to be essentially a therapeutic one in which the infection is limited and reduced in severity.

DISCUSSION

Present information concerning the effect of X-radiation on the immune response and on infection has been reviewed by Taliaferro and Taliaferro (1946). They point out that "ionization occurs only during irradiation, and radioactivity which could influence antigenicity of the antigen is not induced in the body." In the experiments reported here the animals were irradiated 24 hours before infection and it is not unreasonable to assume that the infecting agent, the cholera vibrio, was not directly affected by the prior irradiation of the host. In any case, as Taliaferro and Taliaferro (1946) point out, there is no evidence that ionizing radiations increase virulence or alter antigenicity. The effects of irradiation observed here, then, would appear to be wholly effects upon the host.

Unfortunately for purposes of generalization, the guinea pig is unusually susceptible to the effects of X-radiation, the LD_{50} dose being 170 to 180r in contrast to LD_{50} doses of 700 to 800r for the rabbit and the rat. Whether the

physiological consequences of an LD_{50} dose as it may affect immune or other response to infection are comparable in the guinea pig and, for example, the rabbit is perhaps open to question. However this may be, both harmful and beneficial effects on the infected host have been reported by a number of workers, the harmful effects resulting from large doses of radiation and beneficial effects from small doses. Relative to the former, Taliaferro and Taliaferro (1946) quote an increased susceptibility of guinea pigs to infection with tubercle bacilli resulting from doses of X-ray sufficient to depress the white blood cell count by half, and an increased susceptibility of these animals to intercurrent infection and subsequent pneumonia. Contrariwise, doses of 90 to 95r have been found to increase the resistance of guinea pigs to pneumonia produced by intratracheal inoculation, and similar doses have been found not to reduce susceptibility to *Bacterium coli* peritonitis in the same animal. Without detailed consideration, it will be apparent that the doses of X-radiation used in this investigation cross the threshold from a harmful effect of 150 to 200r, manifested as an increased severity of enteric cholera and in the case of the higher dose as an invasion of the tissues by the cholera vibrio from a focus of infection in the lumen of the bowel, to a beneficial or therapeutic effect of 100r which is apparent as a reduced severity and duration of the enteric infection.

It has been previously shown that both immunity to overwhelming doses of the cholera vibrio and a prophylactic immunity effective in preventing infection with minimal doses are associated with the presence of pre-existing coproantibody. The data reported here are consistent with the hypothesis that coproantibody is an integral and significant part of immunity to this enteric infection and, in fact, provide additional support for it. These observations, taken in conjunction with those on the immune response to parenteral vaccine reported elsewhere (Burrows, Deupree and Moore, 1950), provide a rational explanation for the apparent discrepancy of both beneficial and harmful effects on the host produced by X-irradiation. It seems clear, for example, that the effect of 200r on guinea pigs subsequently infected intragastrically is harmful in that inhibition of coproantibody response after three days allows the enteric infection to increase in severity and at the same time to invade the tissues in generalized form to give rise to septicemia. Here the beneficial consequence of stimulation of coproantibody response is nullified. With reduction of the dose, however, the stimulus of this immune response becomes relatively more important in the entire picture, and adverse indirect effect of a dose of 100r on the infection is clearly apparent and the irradiation becomes therapeutic.

It has been pointed out elsewhere (Burrows, Deupree and Moore, 1950) that (a) coproantibody excretion is inhibited by X-irradiation, but that (b) there is a short period following irradiation, within the first 48 hours, when the immune response is stimulated. The therapeutic effect of appropriate doses of X-radiation described here would appear to be a consequence of a transitory stimulus of the immune response, coupled with only a partial and transitory inhibition of coproantibody. The significant factors are the dosage of radiation and the time at which the antigenic stimulus is provided. Whether this implication can be generalized to other animals and the local

presence of antibody in other infected areas is not clear. The possibility remains, of course, that the protective effect of small doses of radiation lies in the liberation of a natural protective substance from cells destroyed by the irradiation.

It was pointed out earlier that in a sense the symptomless, nonfatal enteric infection of the guinea pig is comparable to the symbiotic relationship of the host and its normal intestinal flora. Present evidence, though limited, suggests that coproantibody, possibly in very low titer, may play a significant role in this relationship, and in that in which a potentially pathogenic bacterium exists in the bowel without causing disease. Such assumptions are consistent with an invasion of the tissues by bacteria of this normal flora following adequate doses of radiation, and may explain in part the role of infection in radiation sickness.

SUMMARY AND CONCLUSIONS

The effect of X-irradiation in doses of 100 to 200r on enteric cholera in the guinea pig was assayed by two methods, autopsy culture of animals sacrificed periodically, and as numbers of vibrios and titer of antibody excreted in the feces.

The period of deaths produced by doses of 150r was associated with generalized bacterial infection in the majority of animals examined, a coincidence consistent with the belief that a bacteremia of intestinal origin is a part of radiation sickness. Of the animals examined at autopsy 63% were so infected, and of these 44% were infected with the cholera vibrio, and the remainder with hemolytic streptococci and staphylococci. Whole body irradiation with X ray in this dosage, then, resulted in a generalized tissue invasion by the cholera vibrio from a focus of infection in the bowel which is not observed in the infected nonirradiated animal.

The course of the infection as assayed by vibrio excretion was also significantly altered by X-irradiation. With doses of 150 and 200r the intensity of the infection was reduced in the first three days following inoculation concurrent with a stimulus of coproantibody response, but subsequently the antibody titer declined and the intensity of the infection increased in the period during which tissue invasion occurred. While the infection was appreciably reduced in intensity in its early stages by doses of 100r, coproantibody response was not effectively inhibited and both intensity and duration of the infection were reduced over that in the nonirradiated animal to give an over-all therapeutic effect of X ray in this dose.

The observed inverse relation between coproantibody titer on the one hand, and the intensity and tendency to generalization of the enteric infection on the other, provides additional support for the hypothesis that effective immunity to enteric infection is in part a function of coproantibody. If it be assumed that natural immunity to bacteria of the intestinal flora is also related to coproantibody response, the inhibition of this immune response by lethal doses of X ray may account for bacteremia of intestinal origin in radiation sickness.

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THE EFFECT OF X-IRRADIATION ON FECAL AND URINARY ANTIBODY RESPONSE

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It has been reported elsewhere (Burrows, Elliott and Havens, 1947; Burrows and Havens, 1948) that enteric infection of the guinea pig with *Vibrio cholerae* produced an immune response, manifested as an excretion of antibody in the feces and urine as well as its appearance in the serum. A similar group of responses is elicited in both the guinea pig and man by immunization with parenterally administered vaccine. Since coproantibody titer reaches a peak and declines while serum antibody titer is still rising, it seems improbable that it represents serum antibody reaching the bowel by diffusion or other less direct means. The data are not inconsistent with a common origin, however, provided it be assumed that: (a) antibody diffuses or is carried from the site of its formation in the tissues in part into the blood and in part into the bowel; (b) dilution in the total mass of tissue, including the blood of the animal, is considerably greater than in the bowel contents; and (c) antibody accumulates, subject to decay, in the blood but not in the bowel. This hypothesis implies an intimate relation between coproantibody titer and rate of antibody formation. Quantitative studies by Koshland and Burrows (1950) have given results consistent with such a relation in that serum antibody titer could be accurately predicted by assuming a rate of formation measured by coproantibody titer, and an accumulation, with normal decay of serum globulin, in the serum. Antibody occurring in the bowel would, then, be newly formed and its transfer from point of origin essentially immediately effective.

Both the site of formation of coproantibody and its mode of transport from the tissues into the bowel are unknown. If it reaches the bowel via mucus and similar secretions, that found in the bowel is perhaps of predominantly local origin, i.e., the cells of the lymphoid-macrophage system (cf. Taliaferro, 1949) in the lamina propria of the intestine. Freund (1927) has shown, however, that antibody is present in the bile of immunized rabbits; this may be taken to suggest that coproantibody is formed primarily in the liver and reaches the bowel by transport in bile. Still another possibility indicated elsewhere (Burrows and Havens, 1948) is suggested by the implied function of lymphatic tissue in antibody formation, beginning with the work of Hektoen in 1915 and most recently reviewed by Craddock and Lawrence (1948), together with migration of lymphocytes through the intestinal mucosa into the lumen of the bowel as described by Bunting and Huston (1921) and others. In this case the lymphocyte would serve as the transport mechanism as well as possibly the site of formation, and on dissolution in the bowel liberate intracellular antibody. These possibilities are not, of course, mutually exclusive and it may well be that more than one is functional.

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The effect of X-radiation on immunity has been reviewed critically and in detail by Taliaferro and Taliaferro (1946). The inhibition of antibody response by relatively large doses of X ray given prior to or in the very early stages of immunization, and its association with radiation-induced lymphopenia and destruction of lymphatic and other tissues, suggested that a study of the coproantibody response in irradiated animals might shed some light on the questions raised above. Furthermore, the inhibition of coproantibody excretion by X-irradiation observed in preliminary experiments suggested that it might play a part in radiation sickness so far as bacteremia of intestinal origin is concerned, and the results of infection studies carried on concurrently and described elsewhere (Burrows, Deupree and Moore, 1950) in turn indicated some of the experiments described here.

METHODS

Young mature guinea pigs weighing 300 to 400 g were used throughout these experiments. They were not pure line stock but inbred in that all were received from a commercial breeder who has maintained a homogeneous stock and whose animals have been used by us over a period of more than 10 years. The animals were given whole body irradiation in doses ranging from 50 to 200 r under the following conditions: 200 KV, 15 milliamperes, 0.5 mm copper and 3 mm bakelite filter, target distance 28.5 inches, roentgen output 17r per minute. In the experiments reported here irradiation followed primary immunization in some instances and in other experiments animals were immunized after irradiation.

The immunizing agent was a cholera O vaccine, prepared as described elsewhere (Burrows, Mather, McGann and Wagner, 1946) from strain NIH no. 41, the Ogawa vaccine strain, which was received from the National Institute of Health in 1942, maintained in lyophilized form, and carried by weekly transplant on blood agar through the present series of experiments. The dose given was 1 mg dry weight, and all animals received 2 doses 4 days apart. In this procedure the height of the immune response is reached about 4 days after the second inoculation.

Feces and urine were collected as 24-hour specimens in a modified metabolism cage with a bifurcated collecting tube. A coarse brass screen set at an angle permitted adequate separation of urine and feces. The latter were collected in 30 ml of physiological saline solution containing 0.5% formalin. Specimens were collected on alternate days, the animals receiving only fresh carrots ad libitum during the periods of collection, and the normal diet of prepared pellets and leafy vegetables the remainder of the time. Groups of 6 animals were used, earlier observations (Burrows, Elliott and Havens, 1947) having shown that variability was adequately reduced by this number. Specimens were not pooled but carried individually; the variability shown in the data here is essentially the same as that observed in other experiments. When relatively large doses of radiation were given, the original group of animals was larger, as 30 animals receiving 200r, and the group of 6 used for collection of specimens augmented by additional animals taken at random from the pool as members of the original group of 6 died. Urine specimens were clarified by centrifugation and preserved by the addition of formalin to 0.5%. Fecal pellets were thoroughly dispersed into a fine suspension in a shaking machine, and clarified by centrifugation, the supernate being used for antibody titrations.

Blood was taken from the heart at intervals of 4 days, allowed to clot, and the serum separated by centrifugation.

Antibody was titrated as agglutinin, using a very light O antigen and incubation at 37 C overnight. Agglutinations were read by indirect light, without mirror or hand lens. The procedure, including details of dilutions, etc., has been described in detail elsewhere (Burrows, Mather, McGann and Wagner, 1946). It may be noted that O antibody was titrated since it has been shown (Burrows, Mather, Elliott and Havens, 1947) that the O antibody is protective while that to the H antigenic complex is not, and hence is presumably that involved in effective immunity to infection. Furthermore, the O agglutinin is apparently identical with the O complement-fixing antibody (Koshland and Burrows, 1950).

RESULTS

As indicated earlier, immunization of the guinea pig with bacterial vaccines, typhoid, dysentery and cholera produces an immune response manifested as the appearance of antibody (agglutinin) in the feces and urine as well as in the serum. The guinea pig responds

relatively rapidly to such antigenic stimulus, peak antibody titer appearing in 5 to 8 days and declining thereafter. The immune response typical of the normal animal is illustrated in figure 1.

Preliminary experiments indicated that a dose of 150r given following immunization with Flexner dysentery cholera vaccine as the immunizing antigen, in which doses of 100r and 150r were given are illustrated in figures 2 and 3. In these the time of irradiation corresponds to the 6th day in the normal immune response shown in figure 1, and it is clear that fecal and urinary titers are markedly affected while serum titer

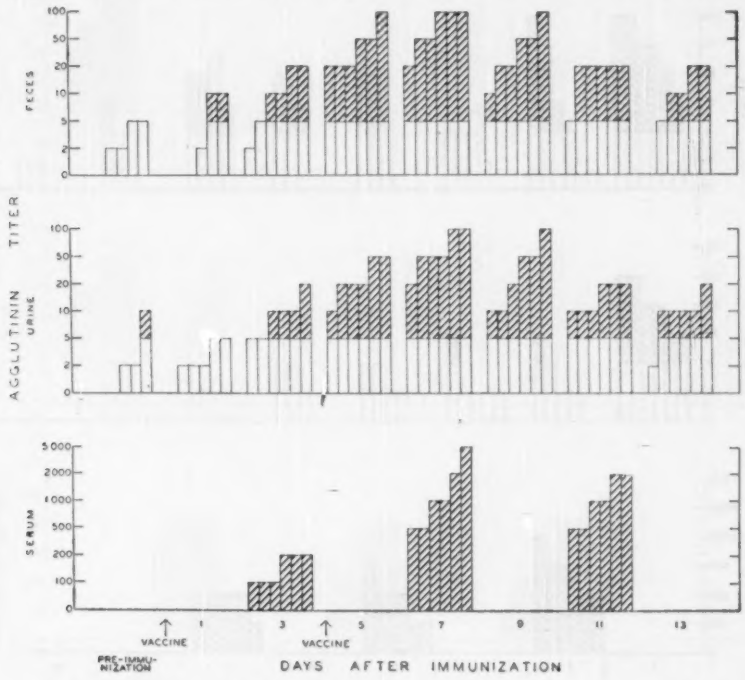

FIG. 1.—The immune response of the normal guinea pig to two doses of 1 mg each of cholera O vaccine measured as fecal, urinary and serum agglutinin. Titers of 1:5 and less in the first two are regarded as not significant and are not crosshatched in this and subsequent figures. Each bar represents an individual animal.

bacillus vaccine and during the period of rise in antibody titer had little or no effect on serum antibody titer, an observation consistent with those of others (cf. Taliaferro and Taliaferro, 1946). The titer of fecal and urinary antibody, however, was sharply reduced and showed no subsequent rise. Essentially similar experiments, using does not appear to be significantly altered. The persistence of detectable titers for a day or two following irradiation is perhaps attributable to secretion of antibody into the bowel and urinary bladder prior to irradiation, actual excretion occurring later. The threshold dosage producing this effect appears to lie in the general vicinity of 100r since

it was not produced by a dose of 50r.

In these, as well as in subsequent experiments described below, individual variation is marked, and some animals were affected while others were not when dosage was near the threshold or critical level. For example, though observation. Theoretically a critical dose could be defined as that inhibiting antibody excretion in half the animals, but definition of significance of low titers is difficult and a dose so calculated is hardly sufficiently precise to warrant its use. Variability in coproantibody

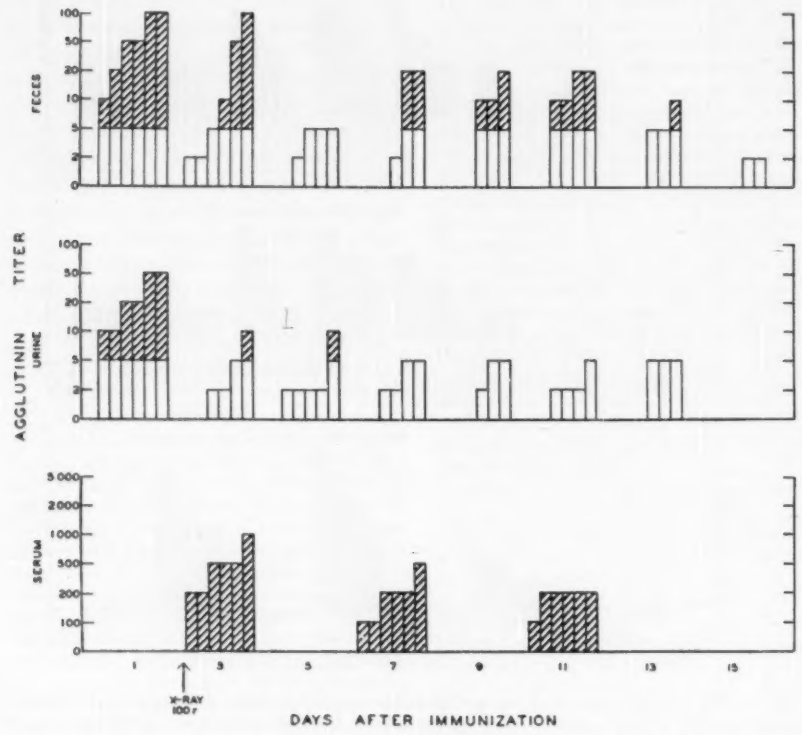

FIG. 2.—The effect of 100r X-irradiation given one day after the second of two inoculations of vaccine and just before the peak in antibody response is reached.

not apparent from the data given in graphical form in figure 2, of the 6 animals in the experiment, 2 showed significant titer of coproantibody 5 days after irradiation, these animals and one additional at 7 days, and the 3 plus one more at 9 days; the other 2 animals were negative throughout the period of response of normals has, it may be noted, essentially the same basis, i.e., some respond well while others do not, rather than differing in time required to attain peak titer in the individual case, though the latter is a factor also.

The effect of irradiation following primary immunization on subsequent

anamnestic response to homologous antigen was examined in a group of experiments in which a booster inoculation of 1 mg of cholera O vaccine by the intraperitoneal route was given 5 days after irradiation and 6 days after the second of the primary inoculations. to completely inhibit fecal and urinary antibody in the experiments described above, appears to depress the anamnestic response in that it is inhibited in some animals; it is not clear whether the apparently greater inhibitory effect on urinary antibody is significantly

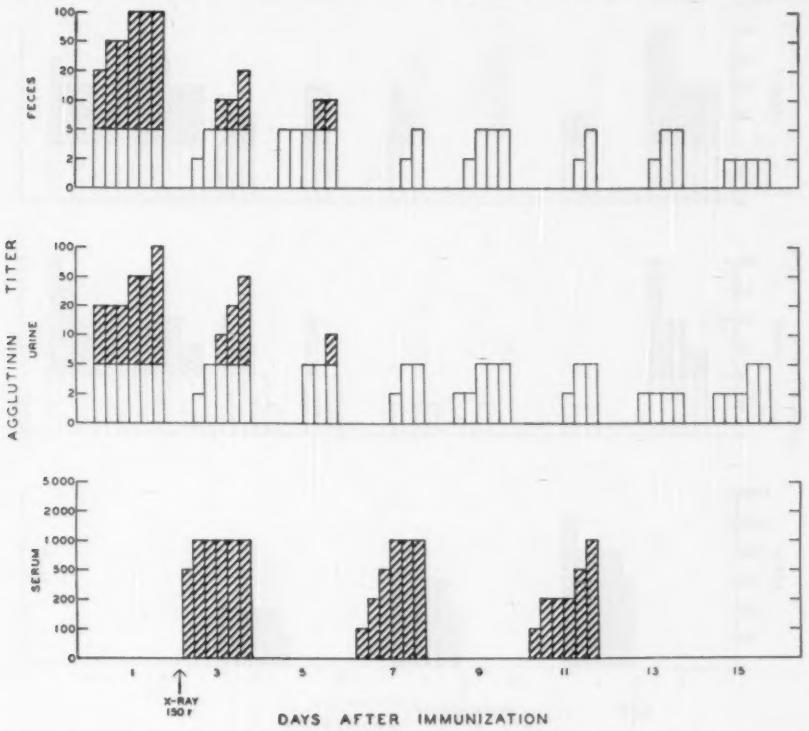

FIG. 3.—The effect of 150r X-irradiation given one day after the second of two inoculations of vaccine and just before the peak in antibody response is reached.

The results of experiments in which doses of 100r, 150r and 200r were given are shown in figures 4, 5 and 6. With a dose of 100r the coproantibody response is possibly slightly reduced over the normal response to primary immunization and is certainly not accentuated as shown by comparison with figure 1. A dose of 150r which sufficed different from that on fecal antibody. With a dose of 200r no fecal or urinary antibody response to reimmunization is apparent. It may be pointed out also that in none of these experiments was there an appreciable serum antibody response to vaccine given after irradiation.

Infection experiments carried out

concurrently and described elsewhere (Burrows, Deupree and Moore, 1950) suggested that an effective coproantibody response occurs in animals infected following irradiation. This possibility was examined further here in a series of experiments in which vaccine was given at varying intervals after irradiation. In one group guinea pigs were given cholera O vaccine, the first dose the day following irradiation, and the second dose 4 days later, i.e., 5 days after irradiation. In the other group the first dose of vaccine was given 3 days after irradiation, and the second 4 days later.

The immune responses to immunization begun the day after irradiation with 50r, 100r and 200r as fecal, urinary and serum antibody are shown in figures 7, 8 and 9. These results clearly indicate that irradiation in these doses stimulates the immune response as coproantibody. With doses as small as 50r coproantibody reached peak titer in 5 days as contrasted with a peak at 7 days in the normal animal as shown by comparison of figure 7 with figure 1, and the second dose of vaccine is less effective in producing an immune response in the irradiated animals. This kind of effect is accentuated with increasing doses of radiation, the peak being reached at 5 days following 100r,

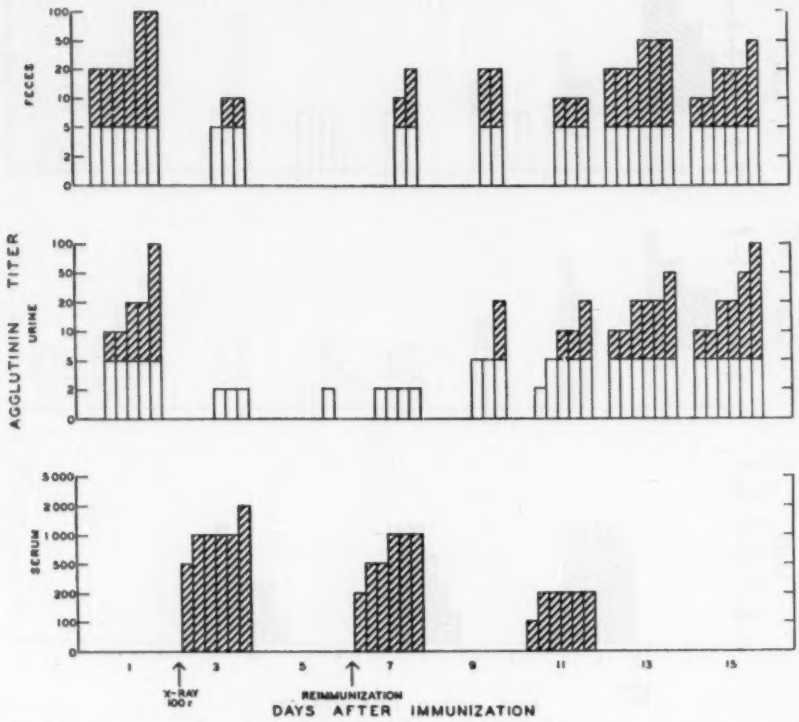

FIG. 4.—The effect of booster inoculation on restoration of fecal and urinary antibody partially inhibited by 100r X-irradiation given after primary immunization.

and on the first day following 200r, and the second dose of vaccine results in no detectable immune response. The rapid decline in fecal and urinary antibody titers, with none remaining by the fifth day after the first inoculation, suggests that the stimulatory effect is only transitory and that subsequent antibody formation is inhibited. It is open to question as to whether there is any inhibition of serum antibody response by 50r, but with larger doses serum antibody response is markedly inhibited. Had the immune response been measured only as serum antibody, the effect of irradiation prior to immunization would appear to be inhibitory only.

In view of the lack of effect of the second inoculation of vaccine in the above experiments, the consequence of delaying immunization until the third day following irradiation with the same doses of X ray, 50r, 100r and 200r, was examined in a second group of animals. The results of these experiments are given in figures 10, 11 and 12. In these experiments a stimulatory effect is still apparent in that peak titers of fecal and urinary antibody are reached earlier than in normal animals, but there is an inhibitory effect manifested as a reduction in the titers reached. These effects also increase with increase in dose of radiation until with 200r the peak

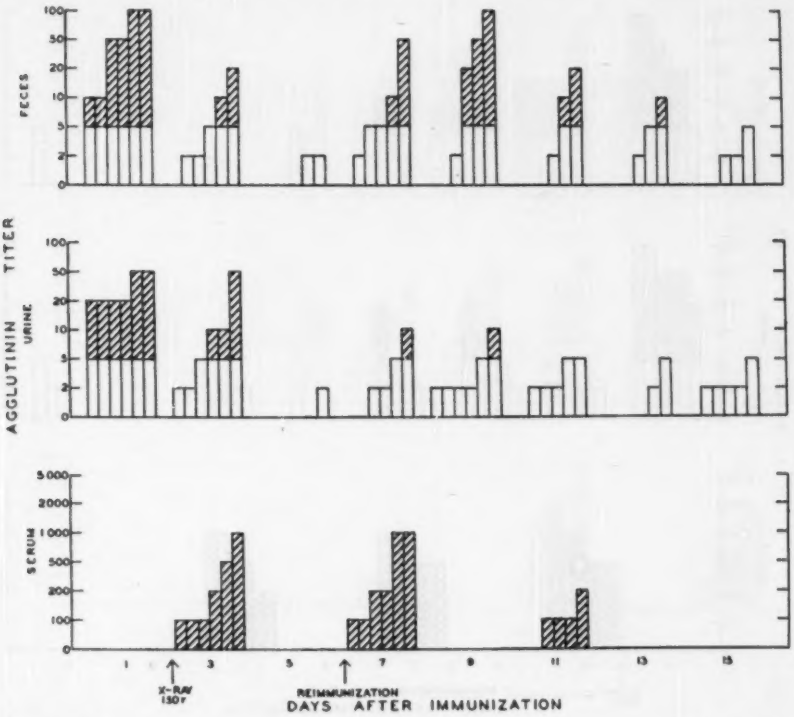

FIG. 5.—The effect of booster inoculation on restoration of fecal and urinary antibody completely inhibited by 150r X-irradiation given after primary immunization.

in coproantibody titer consists of two animals reaching a titer of 1:20. Delay in immunization also results in a greater inhibition of serum antibody response following a dose of 200r in that only one animal showed a transitory titer of 1:100 as contrasted with three showing a titer of 1:100 and one a titer of 1:200 and Taliaferro (1946) have shown, that relatively large doses of ionizing radiation, just sublethal to the host and producing lymphopenia, inhibit the immune response when given prior to or in the very early stages of immunization. When such doses are given after the immune response has begun, anti-

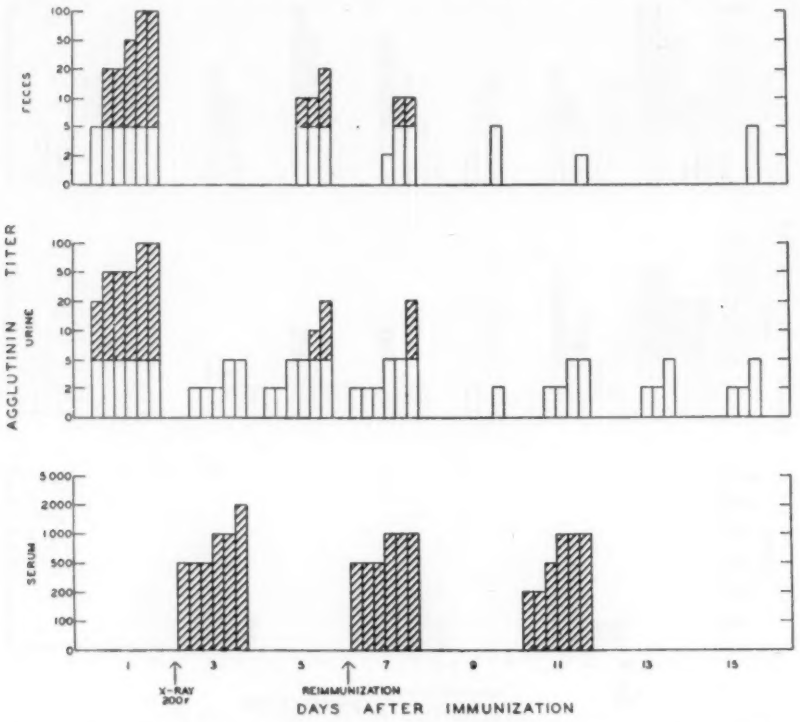

FIG. 6.—The effect of booster inoculation on restoration of fecal and urinary antibody completely inhibited by 200r X-irradiation given after primary immunization.

when immunized immediately after irradiation as shown in figure 9. With smaller doses of radiation the differences, if any, in serum antibody titer associated with delayed immunization are hardly apparent.

DISCUSSION

Published data indicate, as Taliaferro body titer is not significantly affected. Of the more recent work, for example, Craddock and Lawrence (1948) found that doses of 250r given to the rabbit prior to immunization with H-O typhoid vaccine or sheep erythrocytes markedly inhibited the production of agglutinins and antisheep hemolysin, but that the same dose given 4 days after inoculation

did not significantly alter antibody titers to these antigens. It is perhaps of interest also that Evans (1948) has reported similar results in man, patients showing radiation-induced lymphopenia responding only poorly to *Salmonella paratyphi A* vaccine.

Smaller doses of radiation, however, either do not affect antibody response though given prior to immunization, or, as in some reported observations, antibody formation appears to be accentuated. The evidence relevant to the latter has been summarized by Taliaferro and Taliaferro (1946): It has been reported that the production of typhoid agglutinins by the guinea pig is increased by irradiation with 0.5 to 0.75 SED (300 to 450r) prior to immunization; that a 2- to 5-fold increase in immune hemolysin produced by the guinea pig results from prior irradiation with 1 H (125r); and that a 2-fold increase in hemolysin production and a 2- to 5-fold increase in agglutinin, though no increase in precipitin titer, follows immunization after irradiation with 0.25 to 0.5 SED (150 to 300r). Perhaps related are reports of increased resistance of the rabbit to *Bacterium coli* endotoxin and to diphtheria toxin, and both experimental and clinical observations of a therapeutic effect of irradiation on infection. Such beneficial effects can hardly be accounted for on the basis of a selective destruction of the micro-

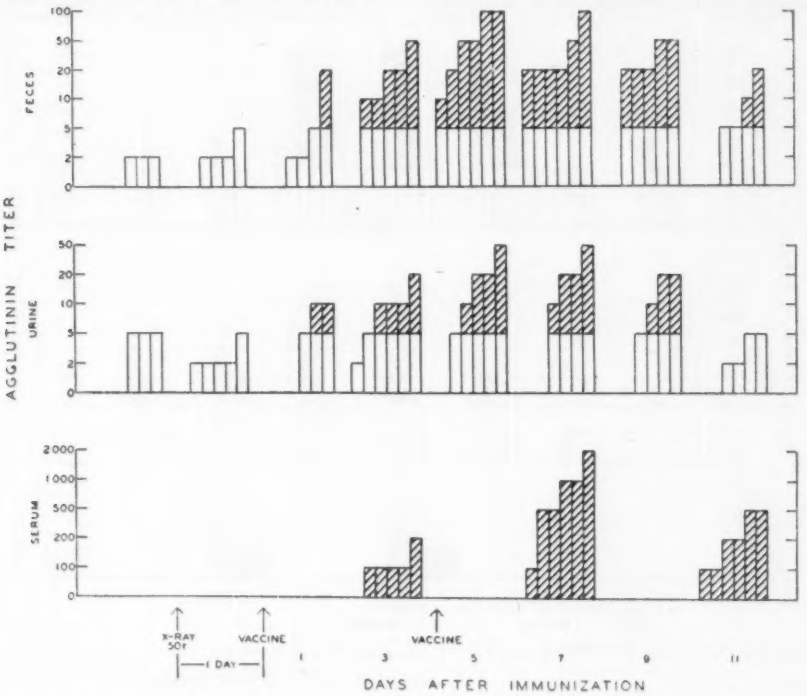

FIG. 7.—The effect of preimmunization irradiation with 50r on the immune response to immunization begun the day following irradiation.

organisms since bacteria are considerably more resistant to the lethal effects of ionizing radiation than the tissues of higher animals (cf. Lea, 1947), and there is no evidence that radiation in these doses alters antigenicity or virulence (Taliaferro and Taliaferro, 1946). It has been suggested that a protective or antitoxic factor is liberated by injured body cells, but the existence of such a factor has not been directly demonstrated. Reported increases in phagocytic index resulting from irradiation prior to immunization may or may not have an antibody basis.

Evaluation of studies on the effect of radiation or other factors on the immune response necessarily includes consideration of the accuracy of antibody titration on the one hand, and individual variation among animals on the other. Agglutinin is commonly titrated by serial dilution of the antibody as $1:2^n$. The subjective element may result in variation of the titer read to one tube on either side of the end point, corresponding to a 2- to 4-fold difference in recorded titer. Thus titer differences must be relatively large and consistent to be significant. Animal variation assumes particular importance at critical or threshold regions, as in X ray dosage in this study, and, as noted above, some animals respond while others do not instead of all responding partially.

If the antibody response is assayed as serum antibody titer, the data reported here are consistent with those

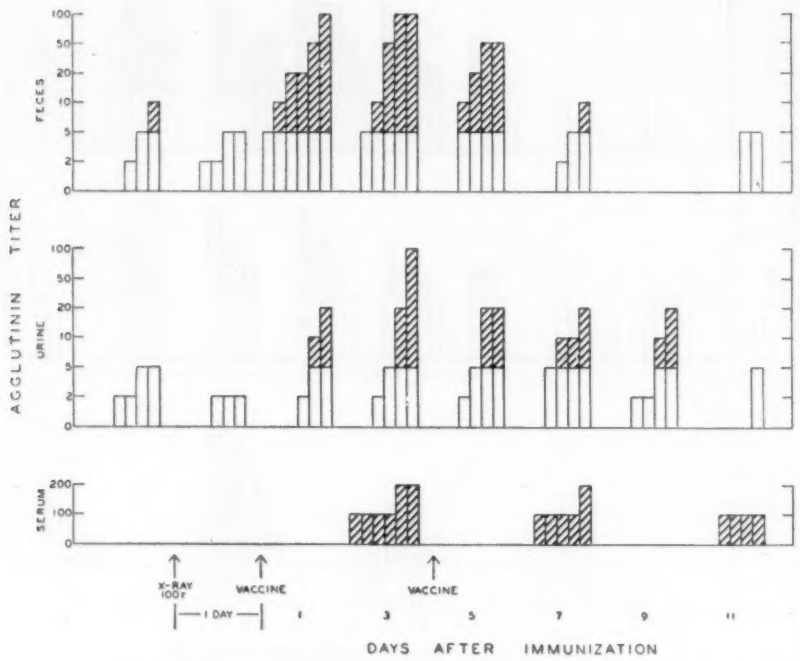

FIG. 8.—The effect of preimmunization irradiation with 100r on the immune response to immunization begun the day following irradiation.

of others in which antibody response is inhibited by preimmunization irradiation but not by postimmunization irradiation. Thus, in all instances postimmunization irradiation in doses as high as 200r did not appreciably alter serum agglutinin titer which declined at a rate not significantly different from that of the nonirradiated animal. In doses of 100 and 200r preimmunization irradiation markedly inhibited serum agglutinin, but not in doses as small as 50r, suggesting that the critical dose lay between 50 and 100r. The failure of serum agglutinin to respond to booster inoculation in animals which had been immunized, irradiated and reinoculated 4 days after irradiation is perhaps consistent with the inhibitory effect of preimmunization irradiation on serum antibody response.

When, however, the immune response was measured as fecal and urinary antibody titer, the effect of irradiation appeared to be dissimilar. Postimmunization irradiation given just prior to peak antibody response had an inhibitory effect, essentially complete with a dose of 150r, and appreciable with one of 100r. Booster inoculation 4 days later was effective in restoring fecal and urinary antibody titer following the incomplete inhibition produced by 100r, and partially effective, that is to say effective in some animals, following the inhibitory effect of 150r. The slight response to reinoculation following a dose of 200r is perhaps of doubtful significance.

The effect of preimmunization irradiation on fecal and urinary antibody response is similarly in contrast to its effect on that of serum antibody. When the first inoculation of vaccine was given

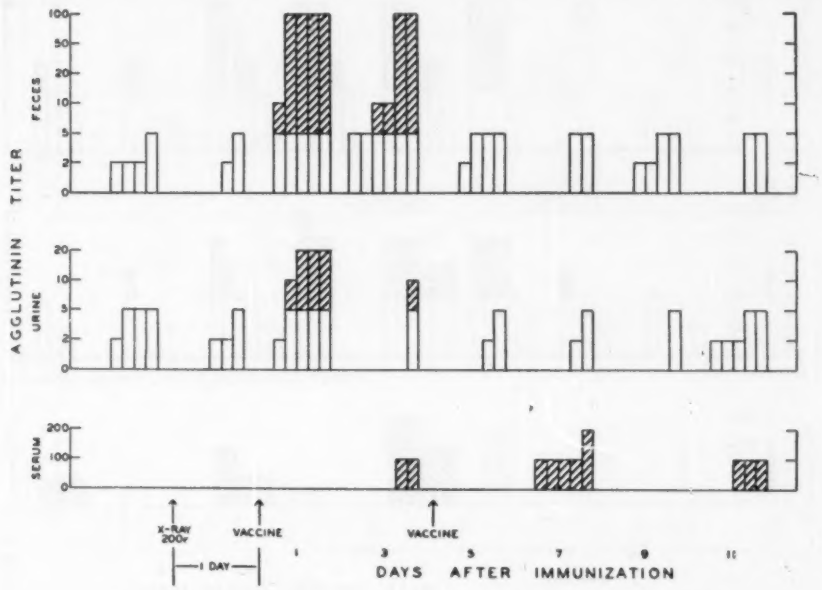

FIG. 9.—The effect of preimmunization irradiation with 200r on the immune response to immunization begun the day following irradiation.

24 hours after irradiation, a dose of 50r was apparently too small to affect the immune response in that the observed titers and rapidity of appearance of antibody were not appreciably different from those of the nonirradiated animal. Some effect was produced by this dose, however, for when the first inoculation was given 3 days after irradiation, some tions gave no detectable immune response. However, when the first inoculation of vaccine was delayed until 3 days after irradiation, the fecal and urinary antibody response was strongly inhibited. These data suggest that appropriate doses of X ray produce a transitory stimulus of the immune response, occurring within the first 72

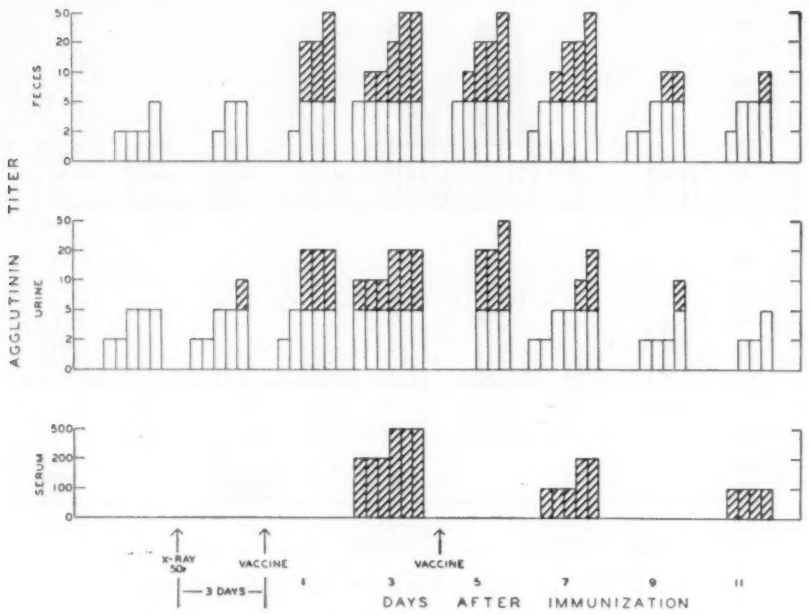

FIG. 10.—The effect of preimmunization irradiation with 50r on the immune response to immunization begun three days after irradiation.

acceleration in the appearance of antibody occurred. With increase in dose to 100 and 200r, the effect of irradiation became more marked. When vaccine was given 24 hours after irradiation at these dose levels, peak titer appeared by the third day with 100r and the first day with 200r in contrast with a peak at 7 days in the normal animal, but disappeared relatively rapidly, and the second of the two vaccine inocula- hours after irradiation, but thereafter the effect of the irradiation is inhibitory. Whether the result is beneficial or detrimental as regards antibody response would seem to be dependent upon the dose of X ray and the time at which antigen is given, the latter possibly including the continued presence of the antigen as in an infection. If these factors are adjusted so that there is a stimulatory effect and the inhibitory

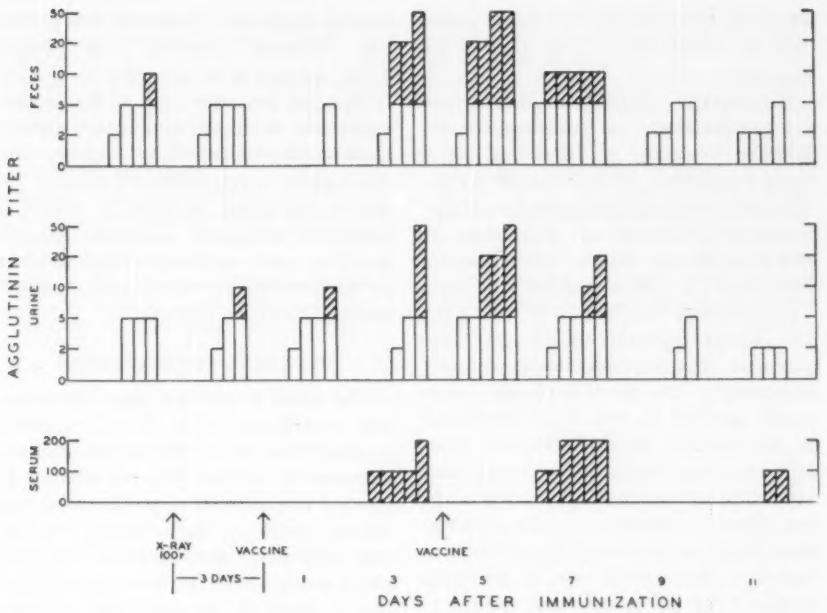

FIG. 11.—The effect of preimmunization irradiation with 100r on the immune response to immunization begun three days after irradiation.

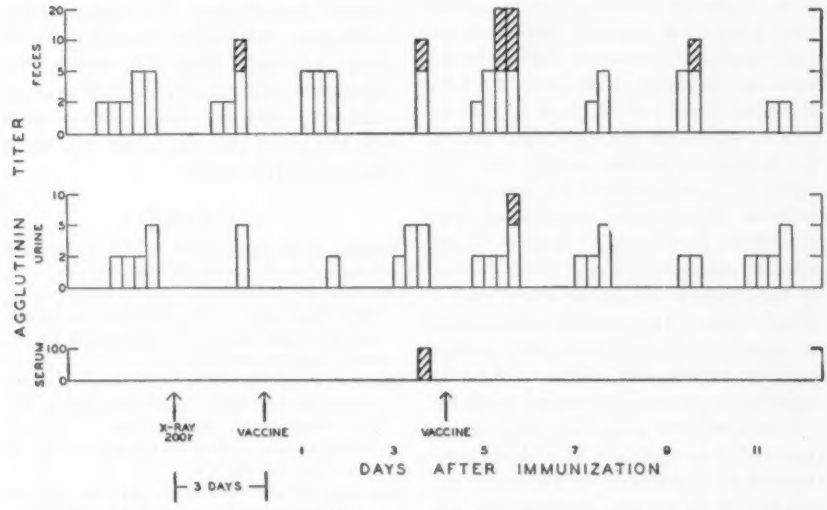

FIG. 12.—The effect of preimmunization irradiation with 200r on the immune response to immunization begun three days after irradiation.

effect is not marked, the consequence may be a total gain in the immune response.

The validity of titration of fecal and urinary antibody as a measure of the immune response is dependent upon some knowledge of their site of formation and mechanisms of secretion. Thus, observed inhibition of titer may be attributable to either inhibition of formation or of secretion or both. Though definitive evidence is lacking, the former appears to be the more probable. For example, since antibody occurring in the feces is clearly but a small portion of the total contained in the animal, its inhibition by postimmunization irradiation can represent inhibition of antibody formation without affecting appreciably the accumulated level in the serum, i.e., that produced at the current rate of synthesis accounts for all of the fecal antibody, but only a part, decreasing as titer increases, of accumulated serum antibody. Similarly, the inferior serum antibody response following preimmunization irradiation possibly represents the small accumulation from the transitory immune response indicated by fecal antibody titer rather than a reduced rate of synthesis over a longer period; for instance, neither serum nor fecal antibody was produced by inoculation delayed three days after irradiation with 200r. Furthermore, both fecal and urinary antibody respond to irradiation of the host in a similar way, and it would appear that possible mechanisms of secretion, into the bowel and urinary bladder respectively, differ at least as regards the organs and tissues involved.

However this may be, the results reported here indicate that local concentration of antibody in the bowel and urinary bladder are affected by appropriate doses of X ray. Concurrent studies on enteric cholera in the guinea pig (Burrows, Deupree and Moore, 1950) suggest that such effects on antibody titer are associated with effective immunity to infection. Taken together, these observations may explain the apparently contradictory results reported by other workers in which a beneficial effect of X-irradiation on infection and antibody response has been observed by some, and a detrimental effect by others.

SUMMARY AND CONCLUSIONS

The effect of pre- and postimmunization irradiation with X ray in doses ranging from 50 to 200r on the immune response of guinea pigs to cholera O vaccine was assayed as serum, fecal and urinary antibody (agglutinin). Irradiation following immunization did not affect serum antibody titer appreciably, but in doses of 100 and 150r partially or completely inhibited the excretion of fecal and urinary antibody, and excretion could be partially restored by booster inoculation. Preimmunization irradiation resulted in sharply reduced serum antibody titer, but produced a transitory stimulus of fecal and urinary antibody, apparent when immunization was begun at one day, but not three days, after irradiation.

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UNUSUAL TYPES OF ENTERIC BACTERIA

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Careful study of large numbers of enteric bacteria brings to light a certain number of cultures which possess biochemical characteristics and antigenic relationships not usually found in association. Such cultures are not easily classifiable in the well-known groups, and genera of the family and their taxonomic position must be determined through mutual agreement of workers particularly interested in the study of Enterobacteriaceae. The purpose of the present paper is to describe a number of such cultures so that their existence is a matter of record and their position within the family of enteric bacteria may be opened to discussion. Inasmuch as the cultures at present are not classified in any of the recognized genera, they will be designated only by symbols applied to them in the laboratory.

1. New Mexico 20 (NM 20)

This culture was isolated in the laboratory of the New Mexico State Department of Health from the stool of a person affected with diarrhea and forwarded to the writers in 1946. On biochemical examination, the culture was found to differ from the usual pattern of the salmonella group only in that it fermented salicin slowly and liquefied gelatin after prolonged incubation.

Serological examination revealed that NM 20 was a diphasic organism and that its major antigens could be identified with antiserums which were at hand. The O antigens were identical with those of members of group D of the genus *Salmonella* (IX,XII). The H antigens of phase 2 were related closely to the nonspecific antigens of *Salmonella*, particularly to the 1,5 phase of *Salmonella thompson*. Absorption of serum for phase 2 of *S. thompson* by NM 20 reduced the titer from 10,000 to 500, and absorption of serum for phase 2 of NM 20 by phase 2 of *S. thompson* reduced the titer from 10,000 to 200.

Thus, so far as the O antigens and phase 2 of NM 20 were concerned, the organism resembled a number of normal salmonella types. However, phase 1 did not resemble any of the antigens previously found in diphasic salmonella cultures. It was related slightly to the salmonella antigens containing the factor g, previously found only in monophasic cultures, the best known of which is *Salmonella enteritidis* (IX, XII: g,m). Phase 1 of NM 20 was agglutinated to 5 to 10% of the titer of salmonella serums which contained agglutinins for antigen g. Likewise, these salmonella types were agglutinated to 5 to 10% of the titer of serum for phase 1 of NM 20. Phase 1 of NM 20 was more closely related to the g,p antigen of *Salmonella dublin* than to any other of the g . . . antigens and was flocculated when tested with large amounts of absorbed single factor p serum. Thus, NM 20 could be expressed by the salmonella formula, IX,XII: g, (p)–1,5. However, phase 1 was much more closely related to the H antigens 13,14 of the Arizona group (Edwards, West and Bruner, 1947), which also are related to the g,p antigens of *S. dublin*. Phase 1 of NM 20 and Ar. 13,14 cross agglutinated to titer, and in absorption

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tests Ar. 13,14 removed all agglutinins from NM 20 phase 1 serum. Therefore, the antigens of NM 20 would be expressed more accurately by the formula Sa.IX,XII:Ar.13,14-Sa.1,5, in which Sa. indicated salmonella antigens and Ar. represented Arizona antigens.

2. Mass. 199 and Mass. 212

These two cultures were isolated in the laboratories of the Massachusetts State Department of Health from stool specimens of two nurses who had no history of enteric disease. They were forwarded to the writers by Dr. R. A. McCready. The organisms also were sent to Beth Israel Hospital, New York City, where Seligmann and Saphra, (1949) found that the O antigens of the cultures were closely related to those of *Salmonella aberdeen* and that the bacteria fermented salicin. These facts were confirmed in this laboratory, where it further was found that with the exception of their fermentation of salicin, the biochemical reactions of the cultures were typical of those of the genus *Salmonella*. It is noteworthy that after several months of cultivation on artificial mediums the avidity of the organisms for salicin was markedly less than when they first were tested. In the later tests, fermentation was delayed greatly, and one culture failed to produce detectable acid. In this respect the organisms resembled other cultures with aberrant biochemical characteristics, notably the sucrose-fermenting culture of *Salmonella typhi* described by Sage and Spaulding (1942).

Reciprocal absorption tests established the identity of the O antigens of Mass. 199 and Mass. 212 and those of *S. aberdeen* (Sa.XI). These O antigens may be expressed by the symbol Ar. 017 since certain Arizona types also possess these antigens (Edwards et al, 1947). The H antigens of the cultures were related closely to those of phase 1 of NM 20, described above, but were not identical with them. They were identical with the Arizona H complex 13,15. Thus these cultures can be represented by the salmonella formula XI:g . . . or more exactly by the Arizona formula 17:13,15. The organisms differ from typical Arizona strains in that they ferment dulcitol and *d*-tartrate but fail to ferment lactose or to liquefy gelatin. It can be said only that the organisms are intermediate types which do not fit exactly into any of the established groups.

3. Diphasic Arizona paracolon bacteria

West, Edwards and Bruner (1947) described a group of cultures which fermented lactose readily, regularly liquefied gelatin, and which displayed typical phase variation of the H antigens. This variation occurred spontaneously and was not observed to differ in any respect from that first described by Andrewes (1922) in the genus *Salmonella*. The biochemical reactions of these cultures were identical with those of the Arizona group with the exception that the diphasic forms fermented *d*-tartrate. Since there was a marked continuity of O antigens in the two groups, the diphasic cultures were classified as members of the Arizona group. Thus, the group of Arizona paracolon bacteria is made up of monophasic and diphasic forms, just as is the genus *Salmonella*. In our experience, the diphasic forms of the Arizona group occur rarely, and the antigenic combinations which they display often are quite bizarre as compared with those which occur regularly in salmonella types. As stated above, the O antigens of many of the cultures are identical with those encountered in monophasic Arizona paracolon cultures, some of which in turn are identical with somatic antigens

found in the genus *Salmonella*. The H antigens of a few cultures are made up of two phases which often occur in conjunction in the salmonella group (e.g., z_{10}-e,n). More often, however, the antigens of both phases are related to those found in phase 1 of normal salmonella types (e.g., a-k), or the antigens of one phase resemble antigens found in salmonella strains while the antigens of the second phase are unrelated to any of those recognized in that genus. Several diphasic lactose-fermenting types have been studied since the original publications of West, Edwards, and Bruner (1947) and Edwards, West, and Bruner (1947). Since some of these cultures are quite closely related to previously described cultures, the antigens of all the diphasic forms recognized are delineated in table 1. For the exact biochemical characteristics of the cultures the reader is referred to previous publications on the group. The newly recognized types differ from previously described cultures in their biochemical characteristics only in that culture Cal. 1141 was an active producer of indol, while Pc 211 was an anaerogenic form which, unlike the other cultures, fermented inositol. Also, it might be said that all of the newly described cultures produced acid and gas from lactose within 48 hours of incubation.

The sources of the newly described cultures were as follows: Cal 1141, Pc 211, Pc 217, and Pc 218—viscera of four gopher snakes (from Dr. W. R. Hinshaw); Minn 97, Minn 98, and Minn 99—livers of three poults of same lot (from Dr. B. S. Pomeroy); Minn 240—liver of poult (from Dr. B. S. Pomeroy); Mich 3—stool, patient with diarrhea (from Mr. Arnold Juenker).

The O and H antigens of these cultures were studied carefully by agglutination and agglutinin absorption. It is not necessary to give detailed tabulated results of these tests but only to comment on the relationships of the antigens to each other and to similar antigens which occur in typical salmonella strains. O antigens 15 and 17 are identical with previously described Arizona O antigens. O antigen 17 is identical with the O antigen of *S. aberdeen*. O antigens 26 and 27 are not related to any previously encountered in the salmonella or Arizona groups.

H antigens 22, 24, 25, and 28 are identical with antigens previously found in diphasic paracolon strains. H antigen 24 is very closely related to antigen r of the salmonella group; the two cross agglutinate to titer. The H antigens represented by the symbol 28 are all very closely related to but not identical with the e,n . . . antigens of salmonella cultures. All lack a fraction of the n complex. When tested with single factor salmonella serums for factors x, z_{15}, z_{16}, z_{17}, and z_{18}, they were agglutinated only by z_{15} serum. The antigens represented by symbol 28 in Pc 110, Pc 217, and Pc 218 are identical, while that in Pc 211 differs slightly from the others.

In the original description of the diphasic strains, it was stated that H antigen 21 was slightly related to antigen a

TABLE 1.—*Antigens of diphasic cultures.*

Strain	O Antigen	H Antigen	
		Phase 1	Phase 2
Pc 48	16 (XXXVIII)	21 (z_{28})	22 (k)
Pc 157	16	21	22
So 57	16	21	22
Pc 166	19	21	22
Pc 158	19	21	22
Pc 195	12 (XVII)	21	23 (1,z_{28})
Pc 155	23	24 (r)	25
Pc 196	24	26	25
Pc 110	25 (XVI)	27 (z_{15})	28 (e,n . . .)
Pc 211	15	27	28
Pc 217	24	24	28
Pc 218	24	24	28
Minn 97	17 (XI)	29 (k)	25
Minn 98	17	29	25
Minn 99	17	29	25
Mich 3	17	29	25
Minn 240	26	29	30 (1,5)
Cal 1141	27	31 (z)	22

Symbols in parentheses indicate salmonella antigens to which the various paracolon antigens are related.

of *Salmonella paratyphia A*. Since that publication, Taylor et al (1948) described *Salmonella chittagong* (I,III,X, XIX: b−z_{35}). Antigen 21 is very closely related to antigen z_{35} of *S. chittagong*, and both are less closely related to antigen a. It is indicated in table 1 that antigens 22 and 29 are both related to antigen k of *S. thompson*. While this is true, the two antigens bear only slight relationship to each other. Antigen 29 is more closely related to k than is 22, agglutinating to 50% of the titer of serum for phase 1 of *S. thompson*. Antigen 30 is identical with phase 2 of *S. thompson* (1,5). Antigen 31 is closely related to phase 1 of *Salmonella poona* (z), and the two cross agglutinate to titer but absorption tests reveal distinct differences in them.

DISCUSSION

The relationships between the various groups and genera of the Enterobacteriaceae are becoming more apparent. As larger numbers of cultures are studied, intermediate or transitional forms appear which cannot be placed readily into previously defined groups. Of necessity, such cultures must be placed arbitrarily in one of the established groups or new groups must be created. Since these intermediate strains form a gradual series of slightly differing types, it is impractical to erect new groups to contain them. Thus, it is necessary to resort to arbitrary placement. *S. chittagong* of Taylor et al (1948), *Salmonella waycross* of Seligmann and Saphra (1948), and *Salmonella weslaco* of Edwards et al (1950) are examples of intermediate cultures which were classified in an arbitrary manner.

In the mind of the worker who is engaged continuously in the study of the antigens of enteric bacteria, it is natural that certain antigenic entities should become associated with rather well defined groups in which these substances frequently occur. The designation of a given antigenic complex as a salmonella antigen or a paracolon antigen obviously is merely a matter of convenience. It is incorrect to assume that any antigen occurs only in one group or genus and does not appear in other groups. While the information concerning continuity of antigens in the enteric bacteria is still fragmentary, it is clear that related or identical antigens may appear in two or more groups.

The discovery of intermediate strains not only creates the problem of their individual placement but makes it increasingly difficult to form lines of demarcation between the established groups. Cultures such as those described above which possess definite antigenic relationships to two groups of bacteria, viz. salmonella and Arizona, yet which possess biochemical properties typical of neither group, emphasize the futility of efforts to establish exact groupings within the Enterobacteriaceae. The present state of our knowledge of the family necessitates its division into relatively narrow groups for practical purposes of classification. Division into groups facilitates study of the bacteria, although as our knowledge increases the groups gradually tend to merge. While these groupings are desirable for immediate purposes of study and cataloging, it must be realized that they are artificial and purely arbitrary. The enteric bacteria form a continuous series of types, and from a scientific standpoint there is no justification for their division into sharply defined groups. It is true that dense centers of population occur within the series, and these comprise the presently recognized genera and groups of the family. However, as knowledge of the family increases the complex relationships among and between the

groups will become more evident, and the true nature of the family as a series of interrelated types will be apparent.

SUMMARY

A number of cultures of Enterobacteriaceae were described which possessed antigenic relationships to the salmonella and Arizona groups. The majority of these cultures were diphasic. The biochemical properties of the cultures were not characteristic of any of the presently recognized genera. Their placement and the classification of enteric bacteria in general was briefly discussed.

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THE COMPARATIVE SUSCEPTIBILITY OF *ANOPHELES ALBIMANUS* AND *ANOPHELES QUADRIMACULATUS* TO A SOUTH CAROLINA STRAIN OF *PLASMODIUM FALCIPARUM*

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Anopheles albimanus is recognized as an important vector of malaria in Central America and in the West Indies. This species occurs also in the Rio Grande valley of southernmost Texas and has been incriminated, upon circumstantial grounds, with malaria transmission there.

Young et al (1946) found that a strain of *A. albimanus* from South Texas was, relative to *Anopheles quadrimaculatus*, nearly insusceptible to imported strains of *Plasmodium vivax*. Boyd et al (1938) and Boyd and Jobbins (1940) found great differences in the relative susceptibility of *A. albimanus* and *A. quadrimaculatus* when both species were fed simultaneously on various strains of *Plasmodium falciparum* and *P. vivax*.

The object of this report is to compare the susceptibility of *A. albimanus* and *A. quadrimaculatus* to a South Carolina strain of *P. falciparum*. This paper is one of a series from these laboratories comparing on a quantitative basis the susceptibility of American *Anopheles* known or suspected to be vectors of malaria.

METHODS

In this study we have used as our standard a strain of *A. quadrimaculatus* which has been cultivated in these laboratories for several years. This strain is designated as our Q-1 strain and was originally derived from the strain domesticated from Florida by Boyd with the possible admixture of Wilson Dam (Alabama) stock, (Young et al, 1946). Our *A. albimanus* strain, designated as A-2, is the strain domesticated by Rozeboom (1936) from Panama and furnished to this laboratory by the Army Medical Center, Washington, D. C.

The strain of *P. falciparum* used was our strain P.f.-5, which was recovered from a Negro living in the vicinity of the Santee-Cooper impoundments in South Carolina during the summer of 1946.

The procedures followed in feeding and handling the mosquitoes have been described earlier (Burgess and Young, 1944). In general, they consisted of feeding mosquitoes of both species (usually about 100 per lot) simultaneously upon a patient, and subsequently incubating the mosquitoes in a temperature-controlled insectary (74–78 F). To ascertain infection intensity and proportion infected, counts of oöcysts were made 12 ± 1 days after feeding. At this time oöcysts were sufficiently large to be counted accurately, but sporozoites were not present in the salivary glands.

To determine the relative rate of development of infections in the mosquitoes, the diameters of the oöcysts were measured and the degree of differentiation recorded for several lots at the time of dissection. To the same end, the time of appearance of the sporozoites in the salivary glands was determined in one pair of feedings.

OBSERVATIONS

Forty-seven pairs of feedings were made, mosquitoes of at least one lot becoming infected in each instance. A larger proportion of *A. quadrimaculatus* mosquitoes was infected in 42 of the 47 feedings. The cumulative data (table 1) show that only one-half as many *A. albimanus* became infected as *A. quadrimaculatus;* however, when the infection rates were high, the difference between the proportions infected was less marked, as can be seen in figure 1.

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A further comparison of the two species on the basis of oöcyst count again showed *A. quadrimaculatus* to be a more susceptible host, the *A. albimanus* mosquitoes consistently showing smaller numbers of oöcysts. Figure 2 plots the number of oöcysts for *A.*

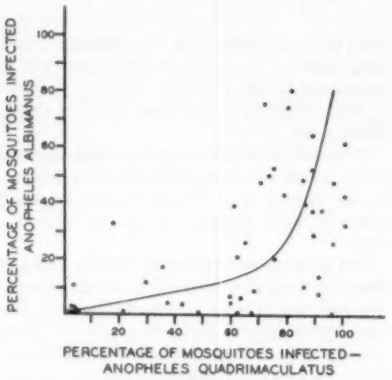

FIG. 1.—A comparison of the percentage infected in 47 lots each of *A. quadrimaculatus* and *A. albimanus* fed simultaneously on the Santee-Cooper strain of *P. falciparum*. Each point on the graph represents a separate pair of feedings and the line indicated was fitted by inspection.

quadrimaculatus against the number for *A. albimanus*. As in the case of proportion infected, when infection intensities were low *A. quadrimaculatus* was, relative to *A. albimanus*, more susceptible than at high infection intensities.

This leads to some difficulty in making a simplified estimate of the relative susceptibility based on oöcyst number. Two methods can be used. In the first place, the mean oöcyst counts for each series of 47 feedings can be compared giving the ratio shown in the fourth line of table 1, that is, that *A. quadrimaculatus* bore 2.5 times as many oöcysts as *A. albimanus*.

The second method is to calculate a ratio of oöcyst means for each pair of feedings and then find the average of the series of 47 ratios (fifth line of table 1). One finds that *A. quadrimaculatus* bore on the average 13.3 times as many oöcysts as *A. albimanus*. This last method gives equal weight to each pair of feedings (except those where one species was uninfected), whereas the first estimate is heavily weighted by the results of the feedings when infection intensity was high. The second method is probably preferable, but the great difference in the two estimates demonstrates that relative susceptibility depends to some degree upon the intensity of the infection and, as a consequence, cannot be too precisely defined.

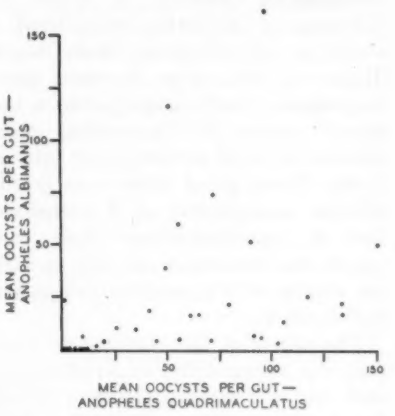

FIG. 2.—A comparison of the mean oöcyst counts in 47 lots of *A. quadrimaculatus* and *A. albimanus* fed simultaneously on the Santee-Cooper strain of *P. falciparum*. Each point on the graph represents a separate pair of feedings. Fourteen points are lost due to crowding near the origin.

With regard to the proportion of mosquitoes which become infected under controlled conditions, *A. quadrimaculatus* again was the more susceptible host. It can be seen from table 2 that *A. albimanus* approaches 70% infected only in the most heavily parasitized lots, whereas *A. quadrimaculatus* seems to approach 90% as an upper limit. It

TABLE 1.—*Summary of 47 paired feedings comparing the susceptibility of* A. quadrimaculatus *and* A. albimanus *to the Santee-Cooper strain of* P. falciparum.

	Anopheles quadrimaculatus	*Anopheles albimanus*	*P* value between species	Index—*quadrimaculatus*/*albimanus*
Number of mosquitoes dissected	1434	1398		
Number infected	826	393		
Percent infected (with S.E.)	58±1.3	28±1.2	<0.0000004	2.1
Mean oöcysts per gut*	38.5	15.5		2.5
Average ratio of mean oöcyst counts†				13.3 (S.E.±2.5) (S.D. 15.2)

* Average of the means of each lot.
† Average of the individual ratios of oöcyst number from 38 lots (9 of 47 lots of *A. albimanus* had no oöcysts; these lot pairs were not included).

would appear, then, that in the case of *A. albimanus* there is a large proportion of inherently refractory mosquitoes, whereas in *A. quadrimaculatus* this fraction is much smaller. This relationship is shown graphically in figure 3. It will be noted that with *A. quadrimaculatus* much higher proportions became infected at low oöcyst levels, whereas *A. albimanus* approached its maximum proportion infected only when the oöcyst level became high (40 or more per gut).

Early in the study it was noted that the oöcysts on a given day appeared smaller in the *A. albimanus* mosquitoes than in the *A. quadrimaculatus*. Careful measurement of oöcyst diameter in 8 pairs of lots showed this difference to be consistent. A series of dissections was then made upon the 12th day of incubation which showed that a significantly larger proportion of oöcysts in *A. quadrimaculatus* had reached the stage of sporozoite differentiation as compared with *A. albimanus*. Similarly a significantly higher proportion of the former mosquitoes had such differentiating oöcysts. These data indicate that the infection develops to maturity more quickly in *A. quadrimaculatus*.

In one series of gland dissections, made during the time sporozoites were entering the glands, a significantly higher proportion of 66 *A. quadrimaculatus* mosquitoes was gland-positive than in a slightly smaller group of *A. albimanus*. This supports the above conclusion that the infections matured faster in the *A. quadrimaculatus*.

In order to determine if the difference in susceptibility of the two species might be due to variation in the production of oökinetes, the oökinetes were counted, following a technique to be described (Eyles, in preparation). No differences were evident. For 16 lots from which counts were made for both species, a mean of 48 oökinetes per 20 minutes was recorded for *A. quadrimaculatus* and 52 for *A. albimanus*, but in 9 of the 16 instances more oökinetes were found in *A. quadrimaculatus*.

Sporozoite indices were not compared, but the ability of *A. albimanus* to transmit this strain of *P. falciparum* had already been demonstrated (Eyles and Jeffery, 1949)

DISCUSSION

The data presented compare the susceptibility of two known vectors of

TABLE 2.—*A comparison of the proportions of* A. quadrimaculatus *and* A. albimanus *mosquitoes susceptible to infections with the Santee-Cooper strain of* P. falciparum *at varying levels of infection intensity as measured by oöcyst count.*

Number of oöcysts per gut	Percent of individuals infected	
	A. quadrimaculatus	*A. albimanus*
1 – 10	38	20
10.1– 20	88	40
20.1– 40	85	55
40.1– 80	81	68
80.1–160	88	69

malaria to a single strain of *P. falciparum*. Such susceptibility data are fundamental to the study of any vector since its capacity for transmission, although greatly influenced by several other factors, is directly dependent upon its susceptibility. Despite the fact that much recent literature points out that most *Anopheles* species are susceptible to malaria infection, there are great variations in the degree of susceptibility, and the present study illustrates a case where one vector is much more susceptible than another to a particular strain of malaria.

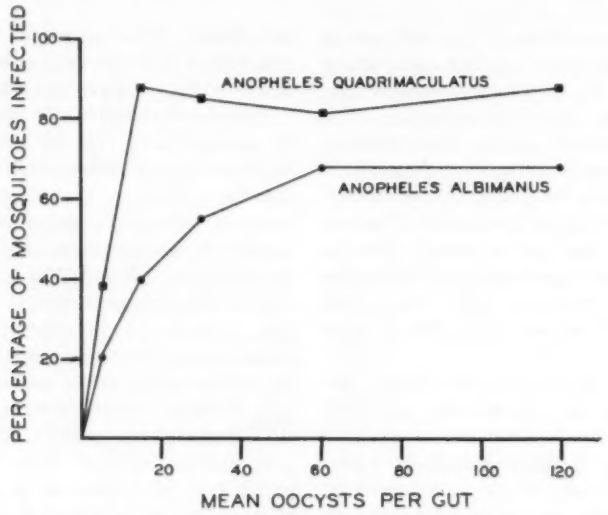

FIG. 3.—The relationship between percentage infected and mean oöcyst count for *A. quadrimaculatus* and *A. albimanus* fed upon patients with the Santee-Cooper strain of *P. falciparum*.

A. albimanus is of especial interest in that the literature, as pointed out in the introduction, indicates that it is variable in its susceptibility to different strains and species of malaria. Boyd and Jobbins (1940) and Boyd et al (1938) fed both *A. quadrimaculatus* and *A. albimanus* on a United States strain of *P. falciparum* (Long strain from Florida). They found that over 7 times as great a proportion of the former species became infected when the 2 species were fed together upon a malaria carrier. No precise data upon oöcyst levels were given.

Our data, while going further in the quantitative sense, conform fairly well with those of Boyd except that in his experiments *A. albimanus* was even less susceptible upon a comparative basis. Such a difference could be due to the larger number of experiments in our study or to the use of different strains of malaria.

In this study, as in Boyd's, it is evident that the coindigenous vector is more highly adapted to the malaria strain than the geographically distant vector. Studies now in progress on a Panama strain of *P. falciparum* indicate that the reciprocal is also true (Jeffery, Eyles, and Young, unpublished observations). This could be the result

of a long coexistence with natural selection, since the greater infectivity would be of survival value to the malaria parasite.

SUMMARY AND CONCLUSIONS

Anopheles quadrimaculatus was found to be more susceptible than *Anopheles albimanus* (Panama strain) to infection with a United States strain of *Plasmodium falciparum*. In an analysis of 47 paired feedings, incubated at 74 to 78 F, the ratio between the proportions infected of *A. quadrimaculatus* and *A. albimanus* was 2 to 1. The average ratio between the mean oöcyst counts was 13 to 1. In heavily infected lots, *A. quadrimaculatus* appeared to approach 90% as a maximum infection rate, whereas *A. albimanus* approached 70%. The development of parasites was more rapid in *A. quadrimaculatus*.

The difference in susceptibility to infection operated only after fertilization of the female gamete, as similar numbers of oökinetes were found in both species of mosquitoes.

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STUDIES IN BACILLARY DYSENTERY

I. FAILURE TO DETECT COPROANTIBODIES IN PATIENTS WITH BACILLARY DYSENTERY (FLEXNER)

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The object of this investigation was to ascertain whether the stools of patients suffering from bacillary dysentery caused by microorganisms of the *Shigella paradysenteriae* group contain agglutinins for the infecting agent—in other words, to demonstrate the existence of coproantibodies in the dysenteric patients.

In bacillary dysentery caused by *Shig. paradysenteriae*, the infecting agent is confined to the lumen of the bowel, where it multiplies and produces the symptoms characteristic of the disease. Positive blood cultures are rare.[1] It is true that a few cases have been reported in which the bacilli invade the deeper tissues and circulate in the blood. However, these patients were all either infants[2–4] or adults of advanced age, probably in preagonal state. Dysentery is essentially a local infection of the intestinal mucosa, in the course of which it has been repeatedly shown that the serum antibodies never reach high values.

Davies,[5] in 1922, published a study of the serological properties of dysenteric stools. The material used came mainly from cases of *Bacterium dysenteriae* (Shiga) infections and, probably, from very severe cases of dysentery, since practically all the specimens contained blood.

Soviet investigators (cited in reference 6) have utilized fecal antibody titration in the early diagnosis of bacillary dysentery. Recently, Harrison and Banvard[7] reported coproantibody excretion during enteric infections. Most of their cases were of *sonnei* dysentery. Burrows, Ell'ott, and Havens[6] studied the excretion of coproantibodies in experimental enteric cholera in the guinea pig.

MATERIALS AND METHODS

Source of the material.—The fecal specimens came from ward (approximately 75-bed capacity) patients of the Insular Psychiatric Hospital. The samples were cultured and those with suspicious growth studied, the cultures showing characteristic biochemical properties of *Shigella* being typed later to establish final diagnosis. Daily specimens of all positive cases were brought to the laboratory.

The patients were followed up for no less than

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30 days after a positive diagnosis had been established. Usually the feces became negative for *Shigella* within the first two weeks after discovery of the infection. When these stools became negative, the search continued for new cases in the same ward or in other wards.

A blood sample was also obtained from each patient with a positive stool, and agglutination tests were performed to determine the serum titer. Twenty-four patients were thus studied.

Procedure.—The specimens were brought to the laboratory in bottles with wide openings, the samples varying from a watery consistency to semisolid or solid; blood was seldom observed in them. The technique for the test was that described by Harrison and Banvard[7] and Burrows et al.[6]

An equal amount of saline by volume was added to each fecal sample, and the glass container was then placed in a Kahn shaker and shaken for about 45 minutes. Cultures were made from this fecal suspension on Shigella-Salmonella agar. This saline mixture was then allowed to extract, in the presence of 0.5% formalin, overnight in the refrigerator. After extraction, 5 ml of supernate (settling had occurred) were pipetted into a centrifuge tube, and centrifugation was carried out in an angle centrifuge. The process was continued until a clear supernate was obtained, which was used for the agglutinin titrations.

Dilutions of this supernatant fluid (1:15, 1:20, 1:30, 1:40, 1:60, etc.) were made in saline in the same way as with serum. In some cases, lower dilutions were made: 1:2, 1:4, 1:8, 1:16. Incubation was carried out at 40 C overnight, and then the racks were refrigerated for several hours, or overnight, before results were recorded. Readings were made from reflections in a mirror, as for Kahn tests, and also in front of a light, using a magnifying glass.

The antigens were prepared from the cultures isolated from the patients, after their agglutinative power had been tested with rabbits' antiserums. The opacity of the antigen dilutions was made to match the No. 2 standard of the MacFarland nephelometer.

The agglutinations for serum titer were performed in the usual manner, starting with a 1:20 dilution and going up to 1:3560 or higher. The antigen used was generally of the same type as that of the organisms isolated from the patients.

7. Harrison, P. E. and Banvard, J. 1947, Coproantibody excretion during enteric infections. Science, **106**: 188–189.

RESULTS

The serum agglutination tests demonstrated that patients had, in most instances, an initial antibody titer when the infection was detected, the value of which fluctuated from 1:40 in some cases to 1:1280 in others. In one or two cases there were no detectable agglutinins in the serum. As a rule, the serum agglutinins increased three or four weeks after the infection was diagnosed. However, there were a few exceptions in which the antibody value did not change.

The tests for fecal agglutinins were performed daily from the time when the infection was detected to approximately 30 days later. The stools were positive for *Shig. paradysenteriae* during one to two weeks after the diagnosis was made. The agglutinations for coproantibodies were always negative. Certain modifications of the procedure, consisting in varying the incubation temperature, more prolonged extraction in the refrigerator, and the use of various dilutions, did not yield different results; and these were consistently negative.

DISCUSSION

As indicated above, observations showed that no agglutinations could be demonstrated in extracts made from bacillary dysentery (*Shig. paradysenteriae*) stools of patients proved to harbor these microorganisms in their intestinal contents. The blood serums of all cases were found to possess agglutinative properties for the Flexner bacilli, the titer of these serums being in some cases as high as 1:1280.

One would probably expect to find antibodies in the lumen of the bowel as a result of the leakage of body fluids through lesions in the mucosa resulting from the infection. In practically all cases studied there was no visible evi-

dence of blood in the fecal matter, which is the general rule in most cases of bacillary dysentery produced by the Flexner bacillus. Occasionally, more severe cases may be encountered, with bleeding into the lumen. However, evidence has been presented by other investigators indicating that antibodies can be secreted and excreted through the intact mucosa of immune subjects. In the cases studied by the writers, even though they showed antibodies in their blood, no evidence could be found to demonstrate that agglutinins had passed through the intestinal mucosa into the lumen of the bowels.

If active immunity is measured by the presence of circulating antibodies in the blood, these patients have a nominal immunity, though an actually effective, prophylactic immunity to enteric infection may not exist. However, several students of enteric infections have indicated the occurrence of a resistance to infection after an attack of bacillary dysentery, even though of short duration.

The possibility of explaining immunity to bacillary dysentery infection, based on the presence of antibody in the bowel, remains conjectural in the absence of supporting evidence. The writers admit, however, that resistance to enteric infection may not depend entirely on "humoral" defenses but that it is conditioned by other factors not demonstrated as yet.

SUMMARY

Coproantibodies in patients suffering from naturally acquired bacillary dysentery infections could not be demonstrated by agglutination tests, using an extract prepared from their feces. The same type of bacterial culture causing the infection was used as antigen, and in some instances the fecal extract was tested against the antigen prepared from the same culture isolated from patients.

Almost all patients studied had agglutinins in their blood for the Flexner type responsible for the infection, and, as a rule, these antibodies tended to increase as a result of the infection.

STUDIES IN BACILLARY DYSENTERY

II. ANTIBODY FORMATION IN *MACACUS RHESUS* MONKEYS AFTER THE ORAL INGESTION OF LIVING AND FORMALIN-KILLED CULTURES OF *SHIGELLA PARADYSENTERIAE*

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Spontaneous bacillary dysentery has been reported in monkeys, though there have been instances of failure to infect them. Bowman[1] reported a spontaneous infection of two captive monkeys with the Flexner type microorganism. Lowell[2] also reported a naturally occurring infection in animals kept in cages. Janota and Dack[3] demonstrated that captive monkeys may be carriers of *Bacterium dysenteriae* (Flexner) and that when kept in a "Vitamin M" deficient diet generally develop bacillary dysentery. Koppisch and Morales-Otero[4] in 1940 studied an epidemic of bacillary dysentery that occurred on a free-range colony of monkeys kept on a small island located five-eighths of a mile from the mainland of Puerto Rico. A concomitant circumstance was a deficiency in the diet. Dack and Petran[5] produced experimental dystentery by introducing Flexner bacilli into isolated segments of the colon of monkeys. They also induced a fatal disease in a normal animal by feeding him a large dose of the same bacterial culture.

The present investigation reports a study on the production of antibodies in normal monkeys (*Macacus rhesus*) after the oral administration of a living culture of *Shigella paradysenteriae* and of a dead vaccine prepared from the same culture.

MATERIALS AND METHODS

Four normal *M. rhesus* monkeys were selected for this experiment. The animals denoted by the letters A and B were adult monkeys that had been caged in the experimental animal house of the School of Tropical Medicine for several years. Monkey A was a large male weighing about 15½ pounds, B, a female animal of about 9 pounds. The other two monkeys (denoted by the letters C and D) were brought to the School for this work from the free-range colony previously mentioned. They were young males weighing about 7½ pounds each.

To monkey A, 10 ml of a 24-hour broth culture of *Shig. paradysenteriae* type I was administered orally through a small rubber catheter. Animals C and D received a similar dose, but of a type III culture. Monkey B was given 10 ml of a 24-hour broth culture of type I microorganisms that had been killed with formalin.

Blood samples for agglutinating tests were taken from all animals before inoculation and these tests repeated every three or four days (table 1). Hemocultures were made from animals A, C, and D before the experiment commenced and repeated every other day, after inoculation, for about 10 days.

Total and differential blood counts were also

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made daily for nine consecutive days of animals A and B, and for three days of monkeys C and D.

Cultures from feces of monkeys A, C, and D were made on Shigella-Salmonella agar. Whenever a suspicious colony was noticed, it was identified to determine whether it belonged to the type of *Shig. paradysenteriae* used to inoculated the animals. Fecal agglutination tests of all 4 animals were performed daily for about 30 days; the same technique described in part I of this paper was used in all tests.

Diarrhea was not observed during the experiment in any of the animals. All animals remained healthy and apparently normal, except monkey A, which refused to eat during the first three days after the culture was given him.

be attributed to the dose of bacterial cultures given was even noted. The highest leucocytic count recorded was 21,200 and the lowest, 8000. The cell counts tended to remain within certain values in the same animal. For instance, monkey C showed a range of 14,000 to 16,000, while in monkey D the figures were 8000 to 10,000.

Nothing significant was observed in the differential counts. The figure for lymphocytes was greater in all cases than the number of segmented poly-

TABLE 1.—*Data from blood samples taken from monkeys given living and formalin-killed cultures of* Shig. paradysenteriae *orally.*

	Date	Total count leucocytes	Hetero-phils	Lympho-cytes	Eosino-phils	Mono-cytes	Baso-phils	Hemo-culture	Plate culture	Serum titer
Monkey A, given 10 ml broth culture (type I)	8–23	21,200	28	67	4	1	0		–	40
	8–24	16,200	25	69	5	1	0	–	+	
	8–25	14,500	31	63	4	2	0		+	
	8–26	14,700	30	64	3	3	0	–	+	
	8–29	14,300	26	68	5	1	0		–	3560
	8–30	16,000	32	62	3	2	1	–	–	
	8–31	13,000	27	69	3	1	0		+	
	9–1	15,000	29	66	5	0	0	–	+	
	9–2	17,000	26	69	4	1	0		+*	3560
Monkey B, given 10 ml formalin-killed broth culture (type I)	8–30	13,600	30	60	8	1	1			80
	8–31	11,400	26	67	5	2	0			
	9–1	16,000	24	70	5	1	0			
	9–2	18,000	27	68	3	2	0			
	9–5	19,200	30	66	2	2	0			160
	9–6	15,500	25	70	3	2	0			
	9–7	12,600	28	65	5	2	0			
	9–8	13,000	23	72	4	1	0			
	9–9	15,000	24	72	2	2	0			
	9–12									320
Monkey C, given 10 ml broth culture (type III)	1–4	16,500	36	57	4	3	0		–	0
	1–5	15,400	34	58	4	6	0	–	+	
	1–6	16,500	31	59	6	4	0		–	
	1–9							–	–	80
	1–11								–	
	1–12							–	–	160
	1–13								–	
	1–16							–	–	160
Monkey D, given 10 ml broth culture (type III)	1–4	9500	33	61	4	2	0		–	0
	1–5	8500	31	63	3	3	0	–	+	
	1–6	8000	30	64	4	2	0		–	
	1–9							–	–	80
	1–11								–	
	1–12							–	–	80
	1–13								–	
	1–16							–	–	40

* Culture of stools were continued for three more weeks, and they were usually positive.

RESULTS

There was a wide fluctuation in values of the total leucocytic counts of each animal throughout the duration of the experiment, but no pronounced deviation from the normal[6] which could morphonuclears, but that seems to be normal for monkeys. Blood cultures were negative throughout the experimental period.

The cultures made from feces were positive for animals C and D but only on the day after oral administration of the living birth culture. Monkey A con-

6. Kracke, Roy. R. 1947, Color Atlas of Hematology, Philadelphia, J. B. Lippincott Co.

tinued to pass the microorganism even after the discontinuation of the experiment, which was 30 days after the infecting dose had been given. The agglutinations with blood serums showed an initial low titer for the strain that was used for producing the infection in the older animals. Monkey A had a titer of 1:40 for *Shig. paradysenteriae* type I and monkey B one of 1:80 for the same culture. The two young animals, C and D, did not show agglutinins in their blood for *Shig. paradysenteriae* type III.

Fecal agglutinins could not be demonstrated in the animals throughout the experimental period.

DISCUSSION

Monkeys and human beings may have "natural" agglutinins in their blood for microorganisms of the *Shig. paradysenteriae* group. During their life, monkeys probably ingest these bacteria, as may be the case with human subjects, and develop specific antibodies, or perhaps the latter may originate from other enteric bacteria having cross-antigens with the bacilli of dysentery.

Dack and Woolpert[7] demonstrated that monkeys contaminated their food with fecal organisms, and later Dack and Petran[5] showed that monkeys in captivity frequently encountered bacilli of the *Shigella* group in their environment.

Two of the animals presented in this experiment showed agglutinins in their blood before they were inoculated with the Flexner cultures. One had a titer of 1:40 and the other of 1:80. These were the older specimens kept in captivity for a number of years (probably 8 to 10). The other two, young monkeys raised on the free-range colony, did not possess agglutinins in their blood.

Only one of the three animals (one of the two old ones kept in captivity) which received a dose of living organism showed signs of infection. It refused to eat for several days, and *Shig. paradysenteriae* type I was isolated from the feces as long as 30 days after inoculation. The agglutinins in its blood increased notably from 1:40 to 1:3560. The other two animals did not show signs of illness, and the bacilli were recovered from their feces only on the day following infection. Agglutinins in the blood rose from 0 to 1:80, in one case, and to 1:160 in the other. This antibody was comparable to that exhibited by the animal which received the dose of dead vaccine, with an agglutination that rose from 1:80 to 1:320, thus tending to indicate that the rise in antibody was due to bacteria that had been killed by the internal defenses of the host and not as a result of actively multiplying bacteria in the intestine.

Furthermore, the observation tends to show that a low antibody titer in the blood was not enough to prevent an infection; that other natural, unknown circumstances played an important role in the resistance to infection. Whether this as yet unknown factor (or factors) concerned with resistance or susceptibility to infection depends on the age of the host or on the condition of the body, which might be associated with an unbalanced nutritional state or certain potentiality of the cells of the mucosa, has to be demonstrated.

When given orally, dead enteric bacterial vaccine apparently induces a slight rise in antibodies.

From the data collected in this experiment, the bacilli of *Shig. paradysenteriae* group (a culture of type I) do not penetrate into the blood stream, even though actively multiplying in the

7. Dack, G. M. and Woolpert, O. 1932, A study of the bacteriology of various levels of the intestine of *Macacus rhesus* monkeys. J. Prev. Med. **6**: 129–139.

intestines. However, a larger number of bacteria in the intestines, or the direct effect of this living microorganism on the intestinal mucosa and its lymphatic system, may be responsible for a greater rise in antibody titer in the blood. Dack and Petran[5] previously proved that agglutinins developed in the serums of these animals when *Bact. dysenteriae* (Flexner) was induced to remain in the bowels under certain experimental conditions.

No agglutinins were detected in the fecal extracts prepared from the experimental animals, not even in the animal the serum of which attained a high value.

The blood counts of all animals were in accord with the value obtained by other investigators. The leucocytic formulas similarly agreed with already proved standards in monkeys. No deviation from these values was noticed during the experimental period.

SUMMARY

Three monkeys were given living cultures of *Shigella paradysenteriae* orally. One was inoculated with Flexner type I and the other two with Flexner type III. The monkey which received type I culture was a mature animal that had been in captivity for many years, and had a low titer of agglutinins in the blood. The other two were young monkeys which had been raised on a free-range colony but showed no agglutinin titer in the blood. The younger animals did not show signs of illness, and their serum antibodies rose only to a significantly low titer.

Dysentery bacilli were recovered from the feces of the mature animal for 30 days after the ingestion of the culture, while a positive fecal culture was obtained from the young animals only on the day following inoculation.

Another monkey received an oral dose of dead *Shig. paradysenteriae* vaccine prepared from a broth culture of a Flexner type I strain. This animal was a mature specimen that had been caged for many years. At the beginning of the experiment it had a low agglutinin titer in the blood. As a result of the ingestion of the bacterial vaccine, a slight antibody serum titer was observed.

The blood counts and the leucocytic formulas of all monkeys remained unchanged throughout the experiment period.

No fecal agglutinins could be demonstrated in these animals.

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